08/182-402

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/50, C07K 3/04, 3/02 C07K 7/10, 13/00, C12Q 1/28 G01N 33/564, 33/573

A1

(11) International Publication Number: WO 93/23073

(43) International Publication Date: 25 November 1993 (25.11.93)

(21) International Application Number: PCT/US93/03837

(22) International Filing Date: 22 April 1993 (22.04.93)

(30) Priority data:
07/885,656 19 May 1992 (19.05.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 07/885,656 (CIP)
Filed on 19 May 1992 (19.05.92)

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF MICHIGAN [US/US]; 475 E. Jefferson, Room 2354, Ann Arbor, MI 48109-1248 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : BAKER, James, R., Jr. [US/US]; 3997 Holdon Drive, Ann Arbor, MI 48103 (US). KOENIG, Ronald, J. [US/US]; 1215 Wynnstone, Ann Arbor, MI 48105 (US).

(74) Agents: LEWAK, Anna, M. et al.; Harness, Dickey & Pierce, P.O. Box 828, Bloomfield Hills, MI 48303 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: THYROID PEROXIDASE EPITOPIC REGIONS

(57) Abstract

Specific eptitopic regions of thyroid peroxidase (TPO), a thyroid-specific membrane autoantigen, have been identified from amino acid 456 to 933, with distinct epitopic regions from amino acid 517 to 630 and from 633 to 933 of the TPO molecule, the former region having at least one distinct binding region therewithin located from aminoacid 592 to 613. The identification and production of these localized epitopes/epitopic regions provide specific diagnostic reagents for autoimmune thyroid disease. As a thyroid-specific immunogenic structure, the TPO epitope/epitopic regions identified can be used for immunotherapy of thyroid disease and thyroid cancer.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

THYROID PEROXIDASE EPITOPIC REGIONS

RELATED APPLICATIONS

This is a continuation-in-part of U.S. Application Serial No. 07/885,656, entitled "Thyroid Peroxidase Epitopic Regions," filed May 19, 1992, herein incorporated by reference.

GENBANK INFORMATION

Thyroid peroxidase sequence has been previously accorded GenBank Accession No. J02969.

FIELD OF THE INVENTION

The present invention relates generally to thyroid peroxidase and, more specifically, to autoantibody epitopic regions of thyroid peroxidase and diagnostic and therapeutic applications thereof.

BACKGROUND OF THE INVENTION

Thyroid peroxidase (TPO) is the major thyroid-specific membrane autoantigen implicated in Hashimoto's thyroiditis, an autoimmune disease characterized by intense lymphocytic thyroiditis resulting in the destruction of the thyroid. Weetman, A.P. et al, *Endocrinol. Rev.* 5:309-355 (1984). TPO's role in autoimmune disease and the precise localization of autoantibody binding has, however, been a subject of much debate. For example, there has been controversy as to whether autoantibodies inhibit thyroid peroxidase activity, thereby contributing to thyroid dysfunction in autoimmune thyroid disease. See Yokoyama, N. et al., *J. Clin. Endocrinol. Metab.* 68:766-773 (1989); Saller, B. et al., *J. Clin. Endocrinol. Metab.* 72:188-195 (1991). Reports on variations in the structure of TPO have suggested alternative mRNA splicing as a source of antigenicity. Kimura, S. et al., *PNAS (USA)* 84:5555-5559 (1987); Nagayama, Y. et al., *J. Clin. Endocrinol. Metab.* 71:384-390 (1990); Zanelli, E. et al., *Biochem. Biophys. Res. Commun.* 170:735-741 (1990). The appearance of these alternative transcripts in normal thyroid glands has, however, brought the relationship of alternative splicing and autoimmunity into question. Elisei, R. et al., *J. Clin. Endocrinol. Metab.*, 72:700-702 (1991). It also now appears that carbohydrate structures are not involved in TPO antigenicity. Foti, D. et al., *Endocrinol.* 126:2983-2988 (1990).

Recent reports on the number and type of autoantibody epitopes found in human TPO have been conflicting. One group has suggested that TPO autoantibodies bind only to conformation epitopes and that no localized epitopes exist. Finke, R. et al., *J. Clin. Endocrinol. Metab.* 71:53-59 (1990). Given that this group demonstrated binding to recombinant TPO produced in CHO cells, but was

unable to show binding to whole recombinant TPO in the bacterial expression system employed, the failure to identify localized epitopes could have resulted from a problem with the expression of intact TPO in bacteria. In contrast, another group has reported the identification of localized autoantibody of TPO epitopes using *in vitro* translated TPO cDNA clones. Libert, F. et al., *EMBO J.* 6:4193-4196 (1987); Ludgate, M. et al., *J. Clin. Endocrinol. Metab.* 68:1091-1096 (1989). Using sera from Hashimoto's disease patients, localized autoantibody binding was identified between amino acids 590 and 675 of the TPO coding sequence. *Id.* While initially identifying only a single antibody binding site in this region, more recent publications of this group indicated the presence of multiple antibody binding sites in the carboxyl half of the TPO molecule. Elisei, R. et al., *Autoimmunity* 8:65-70 (1990); Libert, F. et al., *J. Clin. Endocrinol. Metab.* 73:857-560 (1991). Several other studies using trypsin digests of purified native TPO have also suggested the presence of multiple autoantibody epitopes, including some which appear to be conformational and require disulfide bonds. Nakajima, Y. et al., *Mol. Cell Endocrinol.* 53:15-23 (1987); Yokoyama, N. et al., *J. Clin. Endocrinol. Metab.* 70:758-765 (1990). However, there has been no consensus on the exact number and type of epitopes present in TPO.

The identification of autoantibody thyroid-specific epitopes is important to the understanding and diagnosis of autoimmune thyroid disease and in developing effective immunotherapeutic strategies against thyroid disease and cancer. The identification of specific localized TPO epitopic regions would provide a powerful diagnostic tool for autoimmune diseases such as Hashimoto's to distinguish it from other thyroid conditions. The identification and ability to produce sequences encompassing specific TPO autoantibody epitopes would also be instrumental in developing immunotherapeutic strategies against autoimmune thyroid disease and thyroid cancer.

The development of new therapeutic approaches to treating thyroid cancer is of particular importance. Thyroid cancer is diagnosed in 10,000 individuals in the United States each year. Mazzaferri, E.L. "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990); Cooper, D.S. et al., *Encrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). While modern techniques have led to early diagnosis and treatment, approximately 10% of thyroid cancer patients develop terminal metastatic disease. Robbins, J. et al., *Ann. Intern. Med.* 115(2):133-147 (1991). Thyroid cancer presents an important public health problem in that it most commonly occurs in otherwise

healthy and productive individuals in the third and fourth decades of life. Mazzaferri, E.L. "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990). It is also an extremely important health problem for women, as it occurs three times as often in women than in men. Kramer, J.B. et al., *Adv. Surg.* 22:195-224 (1989). The disease can also be seen in children, especially after radiation exposure. Barnes, N.D., *Horm. Res.* 30:84-89 (1989). While less common in the elderly, thyroid cancer has a much worse prognosis in this population. Mazzaferri, "Thyroid Cancer," Ch. 43:319-313 (Becker K.L., ed.) *Principles and Practice of Endocrinology and Metabolism* (J.B. Lippencott, Philadelphia PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990).

Current therapeutic approaches for the two most prevalent types of thyroid cancer, papillary and follicular carcinoma, involve partial or complete thyroidectomy, often in conjunction with radioactive iodine therapy. However, a substantial number of patients show no response to conventional treatment. For example, some thyroid tumors do not respond to radioactive iodine treatment because the carcinoma is not sufficiently differentiated to concentrate lethal quantities of radioactivity. Additionally, the side effects attending conventional chemotherapeutic and radiation therapy have a serious adverse impact on patient health and well-being.

More sensitive diagnostic and alternative therapeutic strategies against thyroid disease and cancer would thus be desirable. Such strategies can be provided with the identification of specific thyroid peroxidase epitopic regions.

SUMMARY OF THE INVENTION

Specific epitopic regions of thyroid peroxidase (TPO), a thyroid-specific cell membrane enzyme and the autoantibody target in autoimmune thyroid disease, have been identified. The epitopic regions reside from amino acid 456 to 933, with two distinct regions located from amino acid 517 to 630 and from amino acid 633 to 933, the former region having at least one distinct binding region therewithin located from amino acid 592 to 613 inclusive. The latter region also has at least two distinct binding regions therewithin, located from amino acid 633 to 768 and from amino acid 768 to 933. The specific epitopic regions provide a sensitive diagnostic reagent for autoimmune thyroid disease and, as thyroid-specific structures, can be used for immunotherapy of thyroid disease and cancer. Nucleic acid sequences coding for TPO epitopic regions, and sequences complementary thereto, are also contemplated as being within the scope of the present invention.

Since TPO is expressed in most thyroid cancers, patients can be immunized with TPO epitope contained within these regions to induce or augment the immune response to TPO, specifically targeting the TPO-bearing cancer cells. Adoptive immunotherapy involving the harvesting of immune cells from the thyroid, their *in vitro* stimulation by TPO epitope and reimplantation into the patient is also now feasible. Monoclonal antibodies specific for TPO epitope/epitopic region can also be administered for passive immunotherapy.

Autoimmune thyroid disease, such as Hashimoto's thyroiditis, can also be treated by blocking or competing with autoantibody binding to native TPO in the thyroid. More sensitive assays utilizing TPO epitopic binding to screen for the presence of autoantibody are also useful in diagnosing patients with Hashimoto's disease and in distinguishing Hashimoto's from other thyroid disorders.

Other features and advantages of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents immunoprecipitation studies of recombinant thyroid peroxidase (TPO) fragments produced from different length cDNA templates.

Figure 2 summarizes immunoprecipitation results from multiple fragments (TPO constructs) of human TPO.

Figure 3 is a Western blot analysis of the production of immunoreactive recombinant TPO using the pET 3d expression vector.

Figure 4 is a Western blot analysis of MBP-TPO (AA 456-933) fusion proteins isolated from bacterial lysates by amylose affinity chromatography.

Figure 5 illustrates TPO-maltose binding protein products.

Figure 6 is a Western blot analysis of the ability of native TPO to inhibit autoantibody binding to recombinant MBP-TPO fusion protein.

Figure 7 is a Western blot analysis of thyroiditis patient sera reactivity with two different TPO epitopes.

Figure 8 is a block graph illustrating inhibition of Hashimoto's patient sera binding to native TPO in an ELISA by preincubation with different MBP fusion proteins.

Figures 9A and 9B are Western blot analyses of sera from patients with thyroiditis.

Figure 10 is a Western blot analysis of sera from patients with thyroid cancer and thyroiditis.

Figure 11 is an immunoblot analysis of the separation of MBP-TPO fusion proteins by preparative SDS-PAGE.

Figure 12 is a bar graph depicting autoimmune thyroiditis patient T cell proliferation in response to recombinant TPO fragments.

Figures 13A and B are bar graphs depicting proliferation of thyroid cancer patient peripheral blood lymphocytes (PBL) to thyroid antigens.

Figure 14 is a bar graph depicting stimulation of papillary thyroid cancer-infiltrating lymphocytes (CIL) with TPO fusion proteins.

Figure 15 is a Western blot analysis of sera from mice immunized with MBP-TPO (AA 632-933).

Figure 16 is a bar graph showing the proliferation of splenocytes from a mouse immunized with MBP-TPO (AA 632-933).

Figures 17A and 17B are representative Western blots demonstrating autoantibody reactivity to recombinant TPO (AA 513-633).

Figure 18 is a schematic of fine mapping of a TPO epitope using PCR.

Figure 19 is a graphical representation of an ELISA immunoassay for TPO autoantibody epitopes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

IDENTIFICATION OF TPO EPITOPIC REGIONS

As detailed in Specific Example I, several distinct and localized areas of thyroid peroxidase (TPO) have been found to bind autoantibodies of patients with Hashimoto's disease. These autoantibody binding regions were localized from the TPO molecule's C-terminus (AA 933) to amino acid 455, and were recognized by over 90% of the Hashimoto's patients tested. At least one autoantibody epitopic region was located from amino acid 456 to 631, more specifically from amino acid 517 to 630, and another was located from amino acid 633 to 933, the former region having at least one distinct binding region therewithin located from amino acid 592 to 613. (See Specific Example V) Further studies indicated that the latter region also included at least two binding or epitopic regions from amino acid 633 to 768 and from amino acid 768 to 933. The absence of local autoantibody binding in the first 455 amino acids of TPO is remarkable, although the possibility that autoantibodies bind to conformational determinants in TPO involving areas both N-terminal and C-terminal to amino acid 456 cannot be excluded.

Despite difficulties in expressing full-length TPO in *E. coli*, the results of the immunoprecipitation studies described below permitted focusing on the C-terminal half

of this antigen. It was postulated that, due to its smaller size, a construct encoding the C-terminal half of TPO would be more likely to be successfully expressed in bacteria. To further optimize expression, an expression system with a vector (pMAL, New England BioLabs) not previously used in thyroid peroxidase studies was used to produce a bacterial fusion protein, maltose binding protein (MBP). The construct MBP-TPO (AA 456-933) was able to produce full-length fusion protein and many smaller MBP fusion proteins were also produced. The expression of the smaller C-terminal truncated fusion proteins supplemented the Western blot analysis, since the shorter fragments potentially contained fewer autoantibody recognition sites. This appeared to be the case, since some of the smaller fragments reacted with only a subset of the Hashimoto's sera. These results provided evidence that multiple epitopes are present in TPO, and that patients' sera differ in their reactivity to these epitopes.

Several fragments of human TPO recognized by human autoantibodies from a majority of Hashimoto's patients were thus identified. As noted above, these fragments were located in the carboxyl half of the molecule, after amino acid 455, and were recognized in recombinant TPO proteins. At least one autoantibody epitope was located in the region from amino acid 517 to 630, and at least two additional areas of binding were identified within the region from amino acid 633 to 933, more specifically from amino acid 633 to 768 and from amino acid 768 to 933, the former region having at least one distinct binding region therewithin located from amino acid 592 to 613. Autoantibody binding to native TPO could be competitively inhibited by preincubation with these fragments and it appeared there was heterogeneity in the fragments recognized by different Hashimoto's patients.

The TPO fragments recognized by the sera of Hashimoto's patients appear localized to the regions encoded by exons 8, 9, 10, and possibly 11. Banga, J.P. et al., *FEBS Lett.* 266:133-141 (1990). This region has homology with the cytochrome oxidase gene and is potentially involved in TPO enzymatic activity, specifically the distal heme-iron binding region. Kimura, S. et al., *Genet.* 3:113-120 (1988). This suggests a potential relationship between autoantibody binding and TPO function. The TPO fragment where autoantibody binding is localized is also a region where alternative RNA splicing reportedly results in an internal deletion of amino acids 533 to 589. Kimura S. et al., *PNAS (USA)* 84:5555-5559 (1987). The significance of this is not evident, however, as other investigators have found alternative splicing to occur only in regions of the TPO molecule not related to the epitopes identified by the

studies below. Nagayama, Y et al., *J. Clin. Endocrinol. Metab.* 71:384-390 (1990); Zanelli, E. et al., *Biochem. Biophys. Res. Commun.* 170:735-741 (1990). Moreover, since both of the alternative transcripts previously described also appear to be present in normal thyroid glands, the relationship of alternative splicing to the development of autoimmunity is somewhat questionable. Elisei, R. et al., *J. Clin. Endocrinol. Metab.* 72:700-702 (1991).

IMMUNODIAGNOSTIC APPLICATIONS

Immunoassays utilizing isolated natural epitope, the recombinant protein, or a synthetic peptide representing specific TPO epitopic regions can be used to evaluate patients for autoimmune thyroid disease. By screening for antibodies against the TPO epitope or epitopic region, patients with autoimmune thyroid disease such as Hashimoto's can be distinguished from patients with other thyroid disorders, such as Graves' disease.

Current immunoassays for diagnosing Hashimoto's disease use whole thyroid microsomal antigen partially purified from bovine thyroid glands. While this material contains TPO, it is also contaminated with many other bovine proteins and has a slightly different structure from the human antigen. Possibly as a result of such contamination and species dissimilarities, current assays are only about 80% sensitive and do not correlate well with disease activity.

The present invention provides substantially pure TPO epitopic sequences which can be consistently produced in large quantities. As used with respect to the present invention described in the specification and claims, the term "TPO epitopic region, sequence, peptide or amino acid" is meant to include a region or sequence of TPO from amino acid 456 to 933 of SEQ ID NO. 1 or fragment thereof which includes at least one distinct TPO epitope, unless otherwise indicated or evident from the use of the term in context. It will be appreciated that this term thus also includes simply TPO epitope. The term should also be construed broadly as to the source of the TPO epitope or region, whether isolated from natural or recombinant sources or synthetically produced, unless otherwise indicated or evident from the context in which the term is used. By "substantially pure TPO epitopic region, sequence, peptide or amino acid" is meant TPO epitopic region or sequence from any of these sources which is substantially free from undesirable contaminants such as other proteins, with a purity of greater than 90%. The term is not meant to exclude TPO epitope or region which is linked, bound to or intentionally combined with other moieties such as carrier proteins, labels, flanking amino acid sequences and the like. Nucleic acid sequences,

either DNA or RNA, consisting essentially of the coding sequences for the TPO epitopic regions or fragments thereof described above, and nucleic acid sequences complementary thereto, are also contemplated as within the scope of the present invention.

TPO epitopes encompassed by the present invention are specific for thyroid peroxidase and, in substantially pure form, show no measurable cross-reactivity with other types of peroxidase enzymes as seen with whole molecule immunoassays currently in use. The use of TPO epitope as an immunodiagnostic tool in accordance with the present invention thus has the distinct advantage that it is recognized only by antibodies against the specific binding site in human TPO, the actual autoantigen associated with the disease. Furthermore, recombinant or synthetic epitopic regions can be produced in homogenous or substantially pure form without contaminating animal proteins.

As described above and detailed in Specific Example I, the identification of specific TPO epitopic regions and the development of an exemplifying immunodiagnostic assay involved several steps. Prior to this work it was believed that TPO needed to be intact in order to be recognized by antibodies. Using a eukaryotic expression system, it was found that large portions of the TPO coding region could be removed without destroying the antigenic activity. As discussed in Specific Example I, a small segment of the TPO gene coding for approximately 180 of the 933 amino acids in the whole molecule was identified which seemed to identify the antigenic epitope. In order to produce large amounts of this protein for investigation, an expression system using a vector not previously employed in TPO studies was able to translate this specific segment of the molecule. By inserting a small segment of the TPO gene into an appropriate vector, an approximately 140 amino acid fragment of TPO fused to a carrier protein was produced. This fusion protein (MBP-TPO) reacted with human Hashimoto's sera but not with control sera, and inhibited binding to larger portions of the molecule. By using this unique expression system, a recombinant autoantibody epitope was identified and produced.

The further localization of the TPO epitope to a 140 amino acid region between amino acids 517 and 633 results in the ability to produce substantially pure natural or recombinant TPO epitopic sequences or synthetic peptides corresponding to or containing a TPO epitopic region. The specific TPO epitopes of the invention can be used in a variety of immunobinding assay schemes known to those skilled in the art to detect the presence of antibody in clinical or research settings. As described in

therapeutically effective amount of the epitope will be administered for a therapeutically effective duration. By "therapeutically effective amount" and "therapeutically effective duration" is meant an amount and duration to achieve a selected desired result in accordance with the present invention without undue adverse physiological effects, which can be determined by those skilled in the medical arts. It will also be appreciated that administration of the epitopic regions and reagents and vaccines which include the epitopes of the present invention, will be by procedures well established in the pharmaceutical arts, *e.g.* intramuscular, subcutaneous, intradermal and oral administration, either alone or in combination.

Thyroid Cancer. Although there are four types of thyroid carcinomas, medullary, anaplastic, papillary and follicular, the latter two cell types which both arise from the thyroid follicular cells, account for 85% of all thyroid cancers. Papillary cancer accounts for approximately 70% of all thyroid cancers and 85% of radiation-induced cancers. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia, PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). Follicular cancer accounts for 15% of all thyroid cancers, and is seen more commonly in the elderly and in patients with goiter from iodine deficient areas. Block, B.L et al., *Otolaryngol. Clin. North Am.* 23(3):403-411 (1990). Follicular cancer can manifest an aggressive nature despite a benign pathologic appearance, and is more likely than papillary cancer to be metastatic when first diagnosed. Block, B.L. et al., *Otolaryngol. Clin. North Am.* 23(3):403-411 (1990); Watne, A.L. et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991). The overall 10-year mortality of 15 to 50% in patients diagnosed with follicular cancer reflects both the more aggressive nature of this tumor and the poor course seen in elderly individuals with this tumor. Watne, A.L. et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991). In either papillary and follicular carcinomas, larger tumors (>3-5 cm) are associated with poorer outcomes. Watne, A.L. et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991). Smaller lesions can follow a benign and protracted course, but are also found in association with distant metastasis. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia, PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North, Am.* 19(3):577-591 (1990). Thyroid cancers are present *in situ* in approximately 10% of all autopsy specimens, indicating that a benign pre-clinical form of the disease is very common. Bisi, H. et al., *Cancer*

64(9):1888-1893 (1989); Clark, O.H. et al., *Eur. J. Cancer Clin. Oncol.* 24(2):305-313 (1988).

It is important to note that papillary and follicular thyroid carcinoma maintain many of the characteristics of follicular thyroid cells. They display specific proteins associated with thyroid cells, such as the TSH receptor, thyroglobulin and TPO. Mazzaferri, E.L., *J. Clin. Endocrinol. Metab.* 70(4):826-829 (1990); Paul, S.J. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):593-612 (1990). They also function in a manner consistent with thyroid cells, responding to TSH stimulation, concentrating iodine and producing thyroglobulin and thyroid hormone. These features have important implications for treatment and also aid in diagnosis. Tumors that concentrate iodine can be identified and located with ^{123}I or ^{131}I scans, and recurrences of tumors after resection or treatment can be detected by monitoring serum levels of thyroglobulin or by radioactive scanning. Krenning, E.P. et al., *Eur. J. Cancer Clin. Oncol.* 24(2):299-304 (1988); Sisson, J.C., *Endocrinol. Metab. Clin. North Am.* 18(2):359-387 (1989).

Current Therapy for Thyroid Cancer. Papillary carcinoma is often cured by partial or complete thyroidectomy with thyroid hormone replacement therapy to suppress endogenous TSH stimulation of the cancer when the lesion is small (less than 1.5 cm) and well circumscribed. Mazzaferri, E.L., *Semin. Oncol.* 14(3):315-332 (1987). However, in patients over 40 years of age, patients with a history of radiation exposure, patients with multifocal tumors and patients with local lymphatic metastasis, thyroidectomy alone is associated with up to a 40 to 60% recurrence rate in patients with a primary tumor greater than 2.5-3.0 cm. Khan, A. et al., *Throat J.* 68(1):31-32, 34, 39-40 (1989). In these cases and in patients initially presenting with distant metastasis, surgery is followed by adjunctive therapy with radioiodine. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker K.L., ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). While retrospective studies suggest this decreases recurrence rates in many patients, especially in the absence of metastatic disease, some patients have only partial responses to radioiodine and receive a maximal cumulative dose of 800-1000 mCi of ^{131}I without achieving a complete remission. In addition, many metastasis do not concentrate iodine and show no response to ^{131}I administration. This results in a substantial portion of patients (10 to 20%) with papillary cancer who can not be cured by traditional therapy. The overall outlook for these patients is bleak, as no

chemotherapeutic regiment or other treatment modality has been shown to be effective. Knols, L.K. et al., *Semin. Oncol.* 14(3):343-353 (1987). This results in a 10-20% cancer-related mortality for papillary cancer patients in the 20 years following diagnosis. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia, PA 1990); Cooper, D.S. et al. *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990).

Follicular thyroid cancer shares many of the same treatment concerns as papillary cancer, however it is even more lethal. Watne, A.L et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991). Total thyroidectomy for follicular carcinoma without radioiodine therapy does not commonly yield a cure because of the cancer's propensity to widely metastasize. Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). Patients with metastases that do not concentrate iodine tend to have particularly unfavorable outcomes because this tumor is usually more aggressive than its papillary counterpart. Chemotherapy for follicular cancer is also ineffective, and patients often require additional surgery or local radiation for palliation. In older patients with invasive disease, follicular cancer results in mortality rates of 50% in the ten years following diagnosis. Watne, A.L et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991).

Thus, while current therapy for thyroid cancer may be effective, a substantial proportion of patients do not respond to these measures. In these patients, there are currently no alternative therapeutic modalities to relieve their substantial morbidity and mortality. The many patients with thyroid cancer who respond well to conventional treatment obscure the serious nature of this disorder in those patients with progressive disease.

Immune Aspects of Thyroid Cancer. Several findings suggest the immune system attempts to regulate neoplastic thyroid cells. For example, the presence of thyroid cancers *in situ* in 10% of autopsies suggests that neoplastic transformation of thyroid cells is relatively common. Bisi, H. et al., *Cancer* 64(9):1888-1893 (1989); Clark, O.H. et al., *Eur. J. Cancer Clin. Oncol.* 24(2):305-313 (1988). It is possible that a secondary neoplastic event or possibly the breakdown of immune surveillance results in a clinically recognizable thyroid cancer. There is often a lymphocytic infiltrate surrounding papillary thyroid cancer, and this infiltrate can become so intense it must be differentiated from autoimmune thyroiditis. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker K.L., ed.) *Principles and Practice of Endocrinology and*

Metabolism, J.B. Lippencott (Philadelphia PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). In contrast to papillary cancer, follicular cancer does not commonly induce a local immune response (Watne, A.L. et al., *Semin. Surg. Oncol.* 7(2):87-91 (1991)) which may relate to the propensity for this cancer to metastasize. The fact that thyroid cancer is also more progressive and more likely to metastasize in elderly patients (Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990)) may also be a reflection of decreased immune function in this population.

In addition to the local histologic changes, patients with thyroid cancer often have antithyroid antibodies, including serum antibodies against thyroglobulin and thyroid peroxidase. Mazzaferri, E.L., Thyroid Cancer, Ch. 43:319-331 (Becker K.L., ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). Antibodies against these antigens do not seem to alter the prognosis or progression of the cancer and patients with antibodies against the either TPO or thyroglobulin are just as likely as patients without antibodies to die from their tumor. Pacini, F. et al., *Acta. Endocrinol.* (Copenh.) 119(3):373-80 (1988). Antibodies against the TSH receptor are also reported to occur in patients with thyroid cancer. Filetti, S. et al., *N. Engl. J. Med.* 318(12):753-759 (1988). This type of immune response may be detrimental, as anti-TSH receptor antibodies have been reported to cause tumor stimulation in some thyroid cancer patients. Belfiore, A. et al., *J. Clin. Endocrinol. Metab.* 70(4):830-835 (1990). However, the demonstration of antithyroid antibodies in thyroid cancer patients suggests that these patients are able to mount immune responses to a variety of thyroid-associated antigens.

Because serologic immunity is ineffective in many neoplasms and has not appeared helpful in controlling thyroid cancer, cellular immunity might be necessary to suppress thyroid neoplasms. In these patients the induction of antithyroid antibodies require a cellular immune response, and cellular cytotoxic immune responses are reported in tumor infiltrating lymphocytes (TIL) isolated from thyroid cancer. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989). However, the cellular cytotoxic activity of these TIL is not increased compared to the levels seen in peripheral blood and the predominant cytotoxic activity in the thyroid is due to NK cells not directed specifically towards thyroid cells. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989). Importantly, papillary cancer TIL

express lower levels of activation markers than intrathyroidal lymphocytes from patients with thyroiditis. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989); Bagnasco, M. et al., *Clin. Exp. Immunol.* 83(2):309-313 (1991). The thyroid cancer cells themselves do not express ICAM1 and have decreased expression of Class I HLA antigens, indicating a local absence of cytokines (such as γ interferon and TNFα) produced by activated mononuclear cells. Bagnasco, M. et al., *Clin. Exp. Immunol.* 83(2):309-313 (1991); Liaw, K.Y. et al., *Cancer* 46(2):285-288 (1980). Several defects in cytotoxic immune responses have also been documented in the peripheral blood mononuclear cells from patients with papillary cancer, including decreased blastogenic responses to mitogens and additional work has shown a specific defect in peripheral blood NK lysis of thyroid cancer cells. Aoki, N. et al., *Clin. Exp. Immunol.* 38(3):523-530 (1987); Boros, P. et al., *Haematologia* (Budap.) 20(3):189-193 (1987); and Sack, J. et al., *Cancer* 59(11):1914-1917 (1987). Proliferative responses to thyroid antigens are also not as intense in cancer patients as in patients with thyroiditis and are seen in a much smaller percentage of cancer patients. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989). Additionally, non-specific immune stimulation at the time of thyroidectomy has been shown to decrease papillary cancer recurrence rates. Durie, B. G. et al., *Cancer Clin. Trials* 4(1):67-73 (1981). Particular HLA alleles and IgG allotypes (Gm) are associated with the development of thyroid cancer, reinforcing the concept of an immune defect in these patients. (Juhasz, F. et al., *Cancer* 63(7):1318-1326 (1989); Juhasz, F. et al., *Clin. Endocrinol.* (Oxf.) 25(1):17-21 (1986). Thus, while there is evidence of a primary cytotoxic immune response to thyroid cells in thyroid cancer patients, it appears inadequate to control the neoplastic cells.

Chronic Thyroiditis: Cellular Immunotherapy of Thyroid Cancer. The augmentation of cellular immune responses to control thyroid cancer is supported by Hashimoto's (autoimmune) thyroiditis. As discussed previously, this disorder is caused by a specific immune response to thyroid antigens that results in an intense lymphocytic thyroiditis. Weetman, A.P. et al., *Endocrinol. Rev.* 5(2):309-355 (1984). While Hashimoto's is associated with thyroid-specific B cell immune responses, as demonstrated by antibodies to TPO and thyroglobulin (Weetman, A.P. et al., *Endocrinol. Rev.* 5(2):309-355 (1984)), a predominance of thyrocyte-specific T cell responses are observed. Weetman, A.P. et al., *Endocrinol. Rev.* 5(2):309-355 (1984). Importantly, the majority of T cell clones isolated from intrathyroidal cells are $CD8^+$ phenotype and demonstrate cytotoxic activity against thyroid cells, with the end result

usually being hypothyroidism from the complete destruction of the thyroid gland. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989); MacKenzie, W.A. et al., *J. Clin. Endocrinol. Metab.* 64(4):818-824 (1987). The histologic appearance of thyroid glands from patients with end stage thyroiditis demonstrates mainly lymphocytic immune elements and fibrosis. Few, if any, thyroid cells are present and there is total disruption of the normal thyroid architecture. Weetman, A.P. et al., *Endocrinol. Rev.* 5(2):309-355 (1984).

Animal models of thyroiditis reinforce the central role of cellular immunity in the pathogenesis of this disorder. Thyroiditis can be passively transferred between animals by T lymphocytes, T lymphoblasts and thyroid antigen-specific $L3T4^+$ T cell clones, but not with antithyroid antibodies. Okayasu, I., *Clin. Immunol. Immunopathol.* 36(1):101-109 (1985); Charreire, J. et al., *Eur. J. Immunol.* 12(5):421-425 (1982); Romball, C.G. et al., *J. Immunol.* 138(4):1092-1098 (1987); Maron, R. et al., *J. Immunol.* 131(5):2316-2322 (1983). In addition, thymectomy has been shown to prevent the development of the disease. Okayasu, I., *Clin. Immunol. Immunopathol.* 36(1):101-109 (1985). T cells from animals with lymphocytic thyroiditis have been shown to have cytotoxic activity for cultured thyrocytes *in vitro* and $CD8^+$ cells have been demonstrated to proliferate in response to thyroglobulin. Creemers, P. et al., *J. Exp. Med.* 157(2):559-571 (1983); Canonica, G.W. et al., *Clin. Immunol. Immunopathol.* 36(1):40-48 (1985).

Based on the above discussion, by eliciting a cellular immune response to the thyroid which parallels Hashimoto's disease, cytolysis of thyroid cancer cells can be effected. Inducing a "Hashimoto-like" thyroiditis in thyroid cancer patients is desirable for several reasons. The inflammation would be thyrocyte-specific, and thus be unlikely to affect other organs. The immune response would be directed towards micrometastasis which is not detectable by any current assay procedure and would be missed during thyroidectomy. This would provide a means of clearing these cells potentially more effective than ^{131}I and a greater likelihood of cure from thyroidectomy. This approach could also be effective against cancers that did not concentrate iodine, for which there is no present therapy. In addition, while normal thyroid cells would also be destroyed, the effects of hypothyroidism can be easily and completely resolved by treatment and are trivial compared to the effects of progressive thyroid cancer. In any case, all current therapies for thyroid cancer result in hypothyroidism, and immunotherapy offers fewer additional risks than radiation or

surgery. Thus, immunotherapy of thyroid cancer has several advantages when compared to conventional therapy for thyroid cancer.

Several lines of investigation support the efficacy of inducing thyroiditis-like cellular immunity to treat thyroid cancer. While thyroid cancer is reported to occur in patients with Hashimoto's thyroiditis, there are several reasons why augmented antithyroid immune responses would be effective in preventing the development of thyroid cancer. Several studies have reported that thyroid cancer is seen solely in patients with localized thyroiditis surrounding a solitary intrathyroidal mass that was a focus of papillary cancer. Mauras, N. et al., *J. Pediatr.* 106(6):895-898 (1985); Maceri, D.R. et al., *Laryngoscope* 96(1):82-86 (1986). This subgroup of patients may not have had Hashimoto's disease, but in fact had tissue inflammation mimicking thyroiditis as a response to their malignancy. Mazzaferri, E.L., "Thyroid Cancer," Ch. 43:319-331 (Becker, K.L. ed.) *Principles and Practice of Endocrinology and Metabolism*, J.B. Lippencott (Philadelphia PA 1990); Cooper, D.S. et al., *Endocrinol. Metab. Clin. North Am.* 19(3):577-591 (1990). This mislabelling may have arisen from many investigators' identifying thyroid cancer patients with serum antithyroid antibodies as having thyroiditis, a problem with several studies on this subject. Mauras, N. et al., *J. Pediatr.* 106(6):895-898 (1985); Maceri, D.R. et al., *Laryngoscope* 96(1):82-86 (1986). When patients with diffuse goiter or hypothyroidism are considered separately, they have a much lower rate of malignancy than that found in normal patients on autopsy studies. Maceri, D.R. et al., *Laryngoscope* 96(1):82-86 (1986). The few cancers described in patients with generalized lymphocytic thyroiditis are localized and have no regional or distant metastasis. Mauras, N. et al., *J. Pediatr.* 106(6):895-898 (1985); Maceri, D.R. et al., *Laryngoscope* 96(1):82-86 (1986); Hoie, J. et al., *J. Surg. Oncol.* 37(3):147-151 (1988). Given the fact that chronic inflammation is often thought to predispose to the development of cancer, the finding that cancer is seen less often in patients with chronic, diffuse thyroiditis indicates that the specific antithyroid immune response could control neoplastic thyroid cells.

Two groups have previously tried to induce thyroiditis as a treatment for thyroid cancer with limited success. One group used a crude extract of homogenated autologous thyroid cancer to immunize three patients with widely-metastatic thyroid cancer. Amino, N. et al., *Cancer* 36(3):963-973 (1975). Two of the patients were anergic at the time of immunization, and showed no response to either the thyroid cancer or control antigens. The one patient who was not anergic did demonstrate a response to the vaccine and had a 33% decrease in tumor mass that persisted for

over a year. In contrast, more recent studies which used acid-modified thyroglobulin to immunize eight patients with either papillary or follicular cancer were able to induce antithyroglobulin antibodies in five of the patients. Gerfo, P.L. et al., *Surgery* 94(6):959-965 (1983). Two of the patients with thyroglobulin antibodies also developed thyroiditis, with one of the two having a 30% regression of tumor mass. Another patient without documented thyroiditis also had stabilization of the lung lesions (lung metastases were not biopsied) after immunization. Despite this success, antithyroglobulin antibody titers were not high in these patients, and it was difficult to evaluate the degree of immune response to immunization due to interference by circulating thyroglobulin. In addition, considering the present understanding of the pathogenesis of thyroiditis, thyroglobulin may not be the proper target antigen to induce thyroiditis. While neither of these studies demonstrated complete success, they indicate that thyroiditis can be induced in humans through immunization and suggest that the induction of thyroiditis may have an anti-tumor effect on thyroid cancer.

Evaluation of Antigens Potentially Useful In Induction of Thyroid-Specific Immunity in Thyroid Cancer Patients. Given that thyroid cancer regressions have been associated with induced immunity to thyroid cells, and that progressive thyroid cancer is associated with defects in cellular immunity, several methods of inducing and augmenting antithyroid cellular immunity are herein considered. While there are no documented thyroid cancer-specific antigens, it is fortunate that these cancers have been demonstrated to express thyroid-specific antigens. Because these thyroid antigens are human autoantigens, they are known to be immunogenic in man and are associated with human T cell cytotoxicity for thyrocytes. Thus, human thyroid autoantigens would present favorable targets of thyroid cancer-specific immunotherapy, particularly since the normal tissue is essentially expendable.

There are several identified thyroid autoantigens: thyroglobulin, thyroid peroxidase (TPO), the TSH receptor, and more recently, a 64 kDa antigen. Charreire, J., *Adv. Immunol.* 46:263-334 (1989); Weetman, A.P., *Clin. Exp. Immunol.* 80:1-3 (1990); Bech, K. et al., *Clin. Endocrinol.* (Oxf.) 11(1):47-58 (1979); Dong, Q. et al., *J. Clin. Endocrinol. Metab.* 72(6)A:1375-1381 (1991). Each have unique features which may offer advantages if used to induce thyroid-specific immunity. The 64 kDa antigen was recently identified through screening of a thyroid cDNA library and has been shown to be recognized by half the patients with Graves' disease. Dong, Q. et al., *J. Clin. Endocrinol. Metab.* 72(6)A:1375-1381 (1991). It may be a membrane antigen, however there is no evidence that it is associated with destructive immunity to thyroid

cells. In addition, its presence on thyroid cancer cells is unknown, but is reported to be present on other tissues, including muscle cells. Dong, Q. et al., *J. Clin. Endocrinol. Metab.* 72(6)A:1375-1381 (1991). The 64 kDa antigen thus appears to be currently the least viable candidate antigen for immunotherapy.

The TSH receptor is also a membrane antigen specific to thyroid cells. It has recently been cloned and identified as an analogue of the rhodopsin receptor family. Parmentier, M. et al., *Science* 246(4937):1620-1622 (1989). It has a large extracellular N-terminal domain that has unique, potentially antigenic sequences. Wadsworth, H.L. et al., *Science* 249(4975):1423-1425 (1990). However, there may be several problems in using this antigen for immunotherapy. The antigen is not commonly associated with immune responses that lead to destruction of thyroid cells (Burman, K.D. et al., *Endocrinol. Rev.* 6(2):183-232 (1985)) and no evidence of cytotoxic T cell responses is seen in Graves' disease. Antibodies against the TSH receptor may be helpful in that they could block the receptor and prevent stimulation of the cancer by TSH. However, immunizing with this antigen may result in thyroid stimulating antibodies which could stimulate tumor growth. Bagnasco, M. et al., *J. Clin. Endocrinol. Metab.* 69(4):832-836 (1989). Thus, immunotherapy involving the induction of immunity to the TSH receptor above does not appear feasible.

The two thyroid autoantigens with the greatest potential as immunogens for thyroid cancer are thyroglobulin and thyroid peroxidase (TPO), both associated with destructive thyroiditis. Thyroglobulin was the first recognized thyroid autoantigen and the first to be purified. Weetman, A.P. et al, *Endocrinol. Rev.* 5(2):309-355 (1984). It has been closely associated with thyroiditis, and is the major antigen in some animal models of thyroiditis. Okayasu, I., *Clin. Immunol. Immunopathol.* 36(1):101-109 (1985); Charreire, J. et al., *Eur. J. Immunol.* 12(5):421-425 (1982); Romball, C.G. et al., *J. Immunol.* 138(4):1092-1098 (1987); Maron, R. et al., *J. Immunol.* 131(5):2316-2322 (1983); Creemers, P. et al., *J. Exp. Med.* 157(2):559-571 (1983); Canonica, G.W. et al., *Clin. Immunol. Immunopathol.* 36(1):40-48 (1985). However, these animal models of thyroiditis may not closely mimic the disease in man.

The thyroiditis induced by immunization with thyroglobulin is almost uniformly self-limited and does not result in the total destruction of the thyroid gland. Wick, G. et al., *Adv. Immunol.* 47:433-500 (1989); Krco, C.J. et al., *J. Immunogenet.* 17(6):361-370 (1990). In addition, immunization with thyroglobulin does not induce the formation of anti-TPO antibodies, which are seen in both animals with spontaneous thyroiditis and humans with Hashimoto's disease. Antithyroglobulin antibodies do correlate with

disease activity in Hashimoto's patients, but to a lesser extent than anti-microsomal antigen (TPO) antibodies. Weetman, A.P., *Clin. Exp. Immunol.* 80:1-3 (1990). The induction of thyroiditis in animals is also highly dependent on the method of immunization, and only particular strains of animals develop the disease on immunization. Wick, G. et al., *Adv. Immunol.* 47:433-500 (1989). The antigenicity of thyroglobulin is also variable, a phenomenon that in humans and experimental animals seems to be related to the iodine content of the thyroglobulin. This is supported by the observation that thyroiditis is more common in geographic areas where there is dietary replacement of iodine. Sundick, R.S., *Immunol. Ser.* 52:213-228 (1990). Recent work in animals has shown that a T cell epitope in thyroglobulin requires an iodination site. MacKenzie, W.A. et al., *J. Clin. Endocrinol. Metab.* 64(4):818-824 (1987). It therefore may be very difficult to standardize the antigenicity of thyroglobulin. Importantly, thyroglobulin is not a membrane protein, and since it is released from the cells it may act as a blocking antigen that could blunt the induction of immunity or deflect the immune response away from thyroid cells. Thus, although thyroglobulin is associated with destructive thyroiditis in both man and animals, there are several drawbacks in employing this antigen to induce cellular immunity in patients with thyroid cancer.

There are several advantages to the use of TPO as an antigen. In contrast to animal models of thyroiditis induced by immunization with thyroglobulin, spontaneous thyroiditis in both humans and experimental animals is most closely associated with evidence of immunity to TPO. Weetman, A.P. et al., *Endocrinol. Rev.* 5(2):309-355 (1984); Wick, G. et al., *Adv. Immunol.* 47:433-500 (1989). This type of thyroiditis closely mimics the type of immune reaction required to destroy a thyroid cancer cell, since it results in thyrocyte cytolysis and the total destruction of the thyroid. Antibodies to TPO closely reflect the degree of inflammation in the thyroid gland in thyroiditis, and there is T cell sensitization to TPO in most patients. TPO is also a membrane antigen, and therefore presents a better target for immunotherapy than thyroglobulin.

The presence of TPO on most follicular cancers and about one half of papillary cancers has also been documented. Fragu, P. et al., *J. Clin. Endocrinol. Metab.* 45:1089-1096 (1977). It may in fact be present on a higher percentage of thyroid cancers, since many of the older studies measured TPO expression in thyroid cancer by examining tumors for functional peroxidase activity, while more recent immunohistologic studies have identified non-functional TPO in many cancers.

DeMicco, C. et al., *Cancer* 67:3036-3041 (1991). Although TPO resides primarily on the apical membrane and microvillar surfaces in normal thyroid follicular cells, it can be observed on the plasma membrane in inflamed thyrocytes and thyroid cancer cells. Christor, K. et al., *Acta. Histochem.* 58:275-289 (1977). Its expression appears to be induced by local inflammation serving to reinforce the autoimmune responses. Roman, S.H. et al., *Autoimmunity* 2(3):253-263 (1989). These findings indicate that TPO exhibits two important characteristics: it is an antigen associated with cytolytic immunity to thyroid cells and is a membrane antigen associated with a large proportion of thyroid tumors.

Patients with thyroid cancer often show evidence of sensitization to TPO, indicating at least a primary immune response to this antigen. Twenty-five to 50% of patients (depending on the technique) with papillary or follicular cancer have antibodies to TPO, and there have been scattered reports of the documentation of cellular immunity to "microsomal antigen". Pacini, F. et al., *Acta, Endocrinol.* (Copenh.) 119(3):373-380 (1988); Juhasz, F. et al., *Cancer* 63(7):1318-1326 (1989). Unfortunately, until recently many of the studies concerning the immune reactivity to TPO suffered because the identity of the microsomal antigen as TPO was not known, and as a result both the serologic and cellular immune assays were less sensitive than those for thyroglobulin. However, even the results of these older studies support the premise that TPO stimulation of anti-thyroid immunity in patients with thyroid cancer, is advantageous in inducing a secondary immune response rather than a primary one.

Despite the many advantages in using thyroid peroxidase, there are some potential disadvantages. Only one study has shown the effectiveness of affinity purified porcine TPO in the induction of thyroiditis. Kotani, T. et al., *Clin. Exp. Immunol.* 80(1):11-18 (1990). There is also extensive homology between TPO and myeloperoxidase (Kimura, S. et al., *Proteins: Structure, Function & Genetics* 3:113-120 (1988)), and immunization with the whole molecule might induce a cross-reacting immune response leading to the immune destruction of leukocytes, although this is not seen in patients with thyroiditis. Also, TPO may not be present on all thyroid cancers, which may somewhat limit its usefulness.

SPECIFIC EXAMPLE I - TPO EPITOPE IDENTIFICATION AND IMMUNOASSAY

Materials and Methods

Patients. Twenty-five patients with Hashimoto's disease were studied. They were diagnosed because of goiter or hypothyroidism and evidence of anti-microsomal antibodies in a titer greater than 1:1600 as detected with an agglutination assay (Ames

Diagnostics, Ames IA). Study serum was obtained from all patients at the time of their enrollment and was frozen at -70°C until use. Patients with non-autoimmune thyroid disease (multinodular goiters) who had no evidence of anti-microsomal antibodies by agglutination immunoassay were used as controls.

TPO Complementary DNA (cDNA). A Bluescript SK plasmid with the full length (3.1 kb) cDNA for human TPO inserted into the *NotI/XbaI* (5′ - 3′) restriction sites was a gift of Dr. Basil Rapoport, VA Medical Center, San Francisco CA. This plasmid contained the (longest) complete, full length coding sequence and therefore did not include the alternative splicing segments with the internal deletion that had been previously reported in Kimura, S. et al., *PNAS (USA)* 84:5555-5559 (1987); Nagayama, Y. et al., *J. Clin. Endocrinol. Metab.* 71:384-390 (1990) and Zanelli, E. et al., *Biochem. Biophys. Res. Commun.* 170:735-741 (1990).

Immunoprecipitations. Immunoprecipitations of *in vitro* translated TPO were carried out as follows. The Bluescript plasmid containing the cDNA for human TPO was digested separately with *XbaI*, *ClaI*, *Bam*H1 and *BglII* to produce C-terminal truncations yielding translation products of 933 (full length), 631, 455 and 120 amino acids, respectively (designated TPO(1-933), TPO(1-631), TPO(1-455) and TPO(1-120)). The TPO plasmid was also digested with *Eco*47 III and *Bam*HI. The ends of this linearized plasmid were then filled in with Klenow and the plasmid was subsequently ligated to itself. This plasmid was then digested with *XbaI* to yield a translation product with an internal deletion from amino acids 4 to 455 (TPO(Δ4-455)). After agarose gel electrophoresis confirmed digestion of the plasmids, these templates were incubated with T3 RNA polymerase and nucleotide triphosphates for 45 minutes at 37°C to produce sense RNA. Translation was then performed by incubating the RNA with rabbit reticulocyte lysate (Promega, Madison WI) and ^{35}S-methionine according to the vendor's protocol. Translation products were analyzed by SDS-PAGE and autoradiography and were found to have the expected molecular weight before being used in the immunoprecipitations (data not shown). Immunoprecipitation was then performed by mixing 100 µl of a 1:2 dilution of either Hashimoto's or normal sera, or 10 µg of a monoclonal antibody to TPO, with 7500 CPM of *in vitro* translated product for one hour at 37°C. This was followed by the addition 50 µl of Protein A agarose. The agarose was mixed thoroughly for approximately 1 hour at 37°C, and then allowed to settle from the mixture. The supernatant fluid was removed and the agarose was subsequently washed with PBS. The washed agarose was then treated

with sample buffer containing beta mercaptoethanol and SDS to solubilize the bound proteins for subsequent analysis by SDS-PAGE and autoradiography.

Production of Recombinant TPO. The full length coding sequence for TPO was inserted into the *Nco*I restriction site of the *E. coli* expression vector pET 3d using appropriate adapters. See Studier, F.W. et al., *Meth. Enzymol.* 185:60-89 (1990). This construct was grown in *E. coli* HMS174(DE3)pLysS to an OD_{600nm} of 0.4 - 0.5. A small sample was removed for later analysis and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the rest of the culture at a final concentration of 0.4 mM. This culture was grown for three hours at 37°C and the bacteria were harvested by centrifugation (4000 g for 20 minutes) and resuspended in 25 ml of lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25% Tween, 10mM beta mercaptoethanol, 10 mM EDTA, 10 mM EGTA, containing 1 mM PMSF, 0.5 mg/ml leupeptin and 0.7 µg/ml pepstatin A). The treated bacteria preparation was then incubated with 1 mg/ml lysozyme for 30 minutes in an ice bath and sonicated with four, 30 second pulses at the end of the incubation. NaCl was added to bring the salt concentration to 500 mM and the suspension was then spun at 10,000 g for 30 minutes. The supernatant (henceforth called lysate) was saved and brought to a final protein concentration of 2.5 mg/ml using column buffer (10 mM phosphate, 0.5 M NaCl, 1 mM azide, 10 mM beta mercaptoethanol and 1 mM EGTA with PMSF, leupeptin and pepstatin A).

Protein from the bacterial lysate was then analyzed by SDS-PAGE with Coomassie staining, or electro-transferred onto nitrocellulose paper. This transfer was then immunoblotted with the mouse monoclonal antibody against TPO (1 µg/ml) and several high titer anti-TPO sera from Hashimoto's patients (1:100 dilution in phosphate buffered saline (PBS) with 1% BSA) at room temperature, with shaking, overnight. These strips were washed and then incubated with alkaline phosphatase labelled anti-human IgG or anti-mouse IgG, as appropriate for the primary antibody. After a final wash, the blots were developed using BCIP/NBT substrate solution.

A fragment of TPO cDNA was also placed into the *E. coli* expression vector pMalcRI (New England BioLabs, Beverly, MA) in the following manner. The TPO cDNA was digested with *Bam*HI and ligated to an adapter having an EcoRI 5′ end. The ligation product was then digested with *Xba*I and ligated into pMalcRI that had been digested with *EcoR*I and *Xba*I (5′ - 3′). This construct encoded a fusion protein in which the amino terminal portion is maltose binding protein (MBP) minus its leader sequence followed first by the sequence IEGRISEF (one letter amino acid code) and

then amino acids 456-933 of human TPO. This construct was transformed into *E. coli* XL-I and grown at 37°C with shaking until OD_{600nm} readings reached approximately 0.3. IPTG was added at a final concentration of 0.3 mM and the bacteria were cultured for two more hours. The cells were harvested by centrifugation and lysate was produced as described above.

The lysate was run over an amylose affinity column to isolate the MBP fusion proteins according to the vendor's protocol (New England BioLabs), after which the column was washed with 10 volumes of column buffer with 0.05% Tween 20. The fusion protein was eluted using a column buffer containing 10 mM maltose. One ml fractions were collected and assayed for protein by dye binding technique (BioRad, Richmond CA). Fractions containing protein were pooled and analyzed using SDS-PAGE. The proteins were then transferred to nitrocellulose membranes using standard techniques. Western blots of the electrophoresis were performed with 1:100 dilutions of normal sera, sera from TPO antibody positive Hashimoto's patients, and a 1:10,000 dilution of a rabbit antisera to MBP. Binding inhibitions were performed by preincubating the sera with 50 μg of native human thyroid microsomal antigen (prepared as described in Portmann, L. et al., *J. Clin. Endocrinol. Metab.* 61:1001-1003 (1985)) for four hours at room temperature before incubation with the nitrocellulose strip.

ELISA Studies. Ninety-six well microtiter plates were coated with 1 μg/ml anti-TPO monoclonal antibody, 100 μl/well, in bicarbonate buffer, pH 9.6, overnight at 4°C. The plates were washed, and blocked with 200 μl of 1% BSA in PBS for at least two hours. The plates were again washed with PBS, 0.05% Tween 20 (PBST), and incubated with 6 μg/ml human thyroid microsomes (prepared as described in Portmann, L et al., *J. Clin. Endocrinol. Metab.* 61:1001-1003 (1985)) for two hours at room temperature. The plates were then washed and either used immediately or filled with blocking buffer and stored at 4°C.

Hashimoto's patient sera were diluted 1:500 in PBS with 1% BSA, then diluted 1:2 with either the MBP-TPO fusion protein or an equimolar amount of a control fusion protein, maltose binding protein-LacZα. These dilutions were then incubated overnight at room temperature with gentle shaking, before being added to the microtiter plates. The sera were then incubated for four hours at room temperature, after which the plates were aspirated and washed. A 1:1000 dilution of alkaline phosphatase conjugated, anti-human IgG (Sigma, St. Louis MO) was then added and the plates were incubated for two hours at room temperature. The plates were then washed, p-

nitrophenyl phosphate substrate was added and the A_{405nm} was read after 45 minutes.

Results

Immunoprecipitation Studies. ^{35}S-labelled TPO fragments and the full length (933 amino acid) TPO protein were produced by *in vitro* translation and subjected to immunoprecipitation. As shown in Figure 1, six Hashimoto's patients' sera immunoprecipitated the full-length TPO produced from a cDNA linearized with *Xba*I (lanes labeled *Xba*I), but did not precipitate a fragment corresponding to the first 455 amino acids (labeled *Bam*HI for the enzyme used to cut the cDNA). A mouse monoclonal antibody against TPO amino acids 266 to 281 as discussed in Finke, R. et al., *J. Clin. Endocrinol. Metab.* 71:53-59 (1990), was used as a positive control and precipitated both the full-length protein TPO (1-933) and TPO (1-455), but not the smaller fragment TPO ((1-120), labeled *Bgl*II). This confirms that the TPO (1-455) fragment was present and could have been precipitated if the Hashimoto's patients' sera antibodies bound to it. This suggested the absence of local autoantibody binding epitopes in the first 455 amino acids of the translation product.

In order to confirm these studies and further localize putative autoantibody epitopes in the TPO molecule, two additional TPO fragments were produced by *in vitro* translation and immunoprecipitations were preformed. Figure 2 summarizes the results from the monoclonal antibody binding to an epitope from amino acid 266 to 281 (presented in the left column) compared to those from Hashimoto's patients' sera (presented in the right column). The patient sera consistently bound only fragments containing the amino acid sequences after 455. All six Hashimoto's patients immunoprecipitated TPO (1-631) and TPO (Δ4-455). As expected, the mouse monoclonal antibody precipitated TPO (1-631) but not TPO (Δ4-455), the latter having an internal deletion which includes the monoclonal antibody binding site. Taken together, these results suggested the presence of autoantibody epitopes in the carboxyl-half of the TPO molecule, with at least one epitope localized between amino acids 456 and 631.

Studies Using Bacterial Expression Systems. In order to confirm the results of the immunoprecipitation studies using other techniques, *E. coli* were transformed with pET 3d vector expressing the full length cDNA for TPO. The transformed colonies were isolated and grown in culture to an OD of 0.6. The cultures were then split, with half the bacteria being exposed to IPTG. The bacteria were isolated and a crude lysate was produced and subjected to SDS-PAGE (100 μg of protein lane). The gel

was then transferred to nitrocellulose paper, and the paper was immunoblotted with four sera from patients with Hashimoto's thyroiditis and high-titer anti-TPO antibodies (depicted as H1 through H4 in Figure 3) and a monoclonal antibody against TPO (depicted as M anti-TPO). Referring now to Figure 3, Western blots of lysate from pET 3d (TPO) transfected bacteria induced with IPTG (depicted as + lanes) revealed a product which was identified by the mouse monoclonal antibody (referenced with an arrow), but not by the Hashimoto's patient sera. Since the molecular weight of this material was uniformly less than 48 kDa (approximately half the expected molecular weight of TPO) and the mouse monoclonal antibody identified an epitope early in the coding sequence (from amino acids 266 to 281) in the TPO molecule, it appeared that either only the N terminal of the TPO molecule was being translated or that the molecule was being fully translated and the carboxyl half was undergoing proteolytic digestion. Transfection of this vector into protease-deficient bacteria (not shown) did not alter the type or quantity of TPO protein produced, thus suggesting that the short TPO product was due to translation termination rather than proteolytic digestion.

In an attempt to find a more efficient expression vector, pMalcRI (New England BioLabs, Boston MA) containing the coding sequence for MBP, a bacterial protein that binds and transports maltose, followed by the sequence of the insert cDNA nucleotides coding for TPO amino acids 456-933 was used. This construct has three advantages. The bacterial sequence appears to stabilize the subsequent translation of the eukaryotic RNA sequence. The resulting fusion protein can be affinity purified from whole bacterial lysate on an amylose column. In addition, a Factor Xα cleavage site is inserted in the pMALcRI vector between the MBP coding region and the polylinker insertion site so that the fusion protein can be cleaved from MBP to release the protein of interest.

The resulting plasmid (denoted pMAL-TPO 456-933) was transformed into *E. coli* XL1, and the transfected colonies were isolated and grown to an OD of 0.6. The bacteria were induced with IPTG, and cultured for 90 minutes. The cells were then harvested and a crude lysate was produced. This lysate was run over the amylose affinity column. The column was washed and the bound proteins were specifically eluted with 10 mM maltose-containing buffer. These proteins were separated by SDS-PAGE, and transferred to nitrocellulose paper.

The fusion proteins were analyzed for reactivity with Hashimoto's sera using Western blots. Referring now to Figure 4, the lane marked α MBP was probed with a rabbit antisera to MBP. This lane shows all the electrophoresed proteins that were

isolated from the amylose affinity column. The Hashimoto's patient sera react with multiple molecular weight fusion proteins in three general patterns of reactivity (A, B and C). A range of fusion products was revealed from 85 kDa (full-length MBP fused with amino acids 456 to 933 of TPO) to approximately a 42 kDa band (the molecular weight of the MBP without a translated fusion product). See Figure 5. Reactivity with many of the bands above approximately 48 kDa was shown in the Hashimoto's sera, and none of the normal sera showed reactivity. The Hashimoto's patients showed several different patterns of reactivity with the smaller fusion proteins (depicted as dotted and clear arrows in Figure 4), suggesting the presence of unique epitopes in the smaller transcripts that are recognized by sera from different subgroups of these patients. Overall, 24 of 25 Hashimoto's patients with anti-microsomal (agglutination) titers of greater than 1:1600 had positive reactivity to this fusion protein on the immunoblots, but there was no relationship between anti-microsomal antibody titer and immunoblot intensity (data not shown).

The fragments smaller than 85 kDa most likely represent C-terminal TPO truncations rather than N-terminal MBP truncations for two reasons. First, N-terminal truncations of even 10-20 amino acids would not bind to the amylose affinity matrix and would therefore not be isolated in the fractions analyzed by SDS-PAGE (Personal communication, Paul Riggs, New England Biolabs). Second, if there were N-truncations of the fusion protein, the most likely cause would be proteolysis. This latter possibility was addressed by the use of several protease inhibitors (PMSF, leupeptin, pepstatin A and EDTA) and by expressing the fusion protein in the protease-deficient *E. coli* strain SW6AA2 (*lon*, *rpo*H am165). However, neither of these manipulations significantly decreased the quantity of smaller fusion peptides, suggesting they were not proteolytic fragments.

Five of the Hashimoto's patients demonstrating the most intense reactivity with the fusion protein on Western blot were analyzed for cross-reactivity between native TPO and the recombinant MBP-TPO fusion protein. Referring now to Figure 6, plus (+) lanes are blots probed with Hashimoto's sera preincubated with native thyroid microsomes, while minus (-) lanes are the same sera preincubated with a control protein (BSA). In all five sera the antibody binding to the recombinant TPO fusion protein was completely inhibited by preincubation with a ten-fold molar excess of native TPO. This indicated that the epitopes recognized by the patients' sera in the recombinant fusion proteins were present in native TPO.

Because of the possibility that multiple antigenic sites might exist in TPO between amino acids 456 and 933, new pMALcRI constructs were made that expressed smaller portions of the TPO molecule. Specifically, pMALcRI-TPO 456-631 was created, which produced a MBP fusion protein containing TPO amino acids 456-631, and pMAL TPO 632-933 which produces a MBP fusion protein containing TPO amino acids 632-933. The fusion proteins produced by these plasmids were independently recognized by autoantibodies from patients with Hashimoto's disease verifying the presence of multiple autoantibody binding sites in the TPO sequence from AA 456-933. Referring now to Figure 7, Patient 2 not only reacted with the AA 632-933 fragment, but reacted with a wide variety of bands produced from this fragment. Neither patients' binding was inhibited by MBP, confirming the specificity of the antibodies for the TPO sequences. In total contrast, no autoantibody binding could be identified in the N-terminal half (AA 1-455) of the TPO molecule. Maastricht, J. et al., *J. Clin. Endocrinol. Metab.* (accepted for publication 1992). This may be explained by the fact that the N-terminal half of the TPO molecule demonstrates greater homology with other peroxidase enzymes, such as myeloperoxidase, than does the amino acid sequence beyond amino acid 455. See Kimura, S. et al., *Proteins: Structure, Function, and Genetics* 3:113-120 (1988). This suggests that the specificity of the human immune response for TPO autoimmune thyroiditis relates to recognition of sites not shared with myeloperoxidase.

When screening large numbers of patients for reactivity to recombinant peptides corresponding to TPO amino acid sequences 517-630 and 631-933 (see Figures 9A and B), vastly different reactivity is seen. While >80% of patients react to AA 631-933, only 20% of patients react to epitopes located between AA 517-630. However, in these latter patients there is marked reactivity to this region. This suggests unique reactivity in patients with thyroiditis, and potential differences in reactivity which may relate to disease pathogenesis.

It also appears that the region between amino acids 633 and 933 contains more than one antibody binding site. This is supported by data indicating that only half of the patients who bind to the amino acid 633 to amino acid 933 fragment also bind to a peptide fragment corresponding to amino acids 633 to 768. This indicates that the patients who do not bind to the 633 to 768 fragment bind to a region involving amino acids 768 to 933.

ELISA Inhibition Assays. Figure 19 shows a graphical representation of an ELISA for detection of human antibodies against TPO epitopes. Plates are coated

with monoclonal antibodies directed against MBP and partially purified MBP-TPO fusion protein is incubated, and bound by the monoclonal antibody. After washing, human sera diluted 1:400 is added to the well and binds to the TPO epitope (to prevent sera binding to bound MBP, the sera is preincubated with soluble MBP). The bound human antibody is then detected with anti-human IgG preabsorbed against mouse IgG. An enzyme substrate is then added to quantitate bound enzyme, which is proportional to the amount of bound human IgG. The assay can be adapted to measure antibody to any TPO epitope (or any other autoantigen) by substituting a different MBP-containing fusion protein).

ELISA inhibitions were carried out with seven patients having anti-TPO activity. The solid phase antigen used in the assay, of which the results are set forth in Figure 8, is native human TPO bound to the ELISA plate wells using the mouse monoclonal anti-TPO antibody. As shown in Figure 8, preincubation with the MBP-TPO (AA 456-933) fusion protein (depicted as striped bars) was compared to inhibitory binding to native TPO as compared with preincubation with a control fusion protein (MBP-LacZα, depicted as solid bars). Preincubation with the MBP-TPO fusion protein inhibited binding to the native TPO 70-100% as compared to the control fusion protein which caused only 10-15% inhibition. In four of the eight patients, the inhibition with MBP-TPO was to the background levels of the assay. These results suggest that the majority of the autoantigen epitopes in native TPO are located in the area of the region of the molecule distal to amino acid 455.

SPECIFIC EXAMPLE II - IMMUNOTHERAPY

It is important to evaluate autoantibody reactivity to the newly identified localized epitopes of TPO because of the prevalence of antibodies to the thyroid microsomal antigen in several diverse patient populations. Elderly patients without evidence of overt thyroid disease, where up to 20% of individuals (primarily females) demonstrate autoantibodies as described in Spaulding, S.W., *Endocrinol. Metab. Clin. North Am.* 16(4):1013-1025 (1987), were the first individuals evaluated to determine if some of these patients had antibodies against the localized TPO determinants as a marker of true autoimmune disease. During the course of these studies sera from three patients with thyroid cancer were evaluated. The Western blots of Figure 10 depict studies of a patient with papillary thyroid cancer (Patient A), follicular thyroid cancer (Patient B) and Hashimoto's thyroiditis (Patient C). Reactivity with both types of fusion proteins and human TPO (lanes A, B, and C), as well as the lack of reactivity with MBP (lane D), is shown. While these patients were not actually "normal" elderly

individuals, all three demonstrated autoantibodies to the recombinant TPO epitopes. It appeared that these patients' antibodies were in lower titer than those found in most Hashimoto's patients. However their reactivity to the localized TPO epitopes was as great or greater than that seen to the whole, native TPO molecule. The fact that cancer patients would have antibodies to TPO despite clinically significant and locally progressive thyroid cancer appears paradoxical. However, none of these three patients, one with follicular cancer and two with papillary carcinoma, exhibited distant metastasis. Both of the patients with papillary cancer had a mononuclear cell infiltrate surrounding their cancers but still had evidence of local progression. Thus, there was no clear relationship between the presence of anti-TPO antibody and either the type of thyroid cancer or evidence of a local immune reaction. This suggested that patients with thyroid cancer had pre-existing immune responses to TPO and that antibody to TPO alone might not be sufficient to control thyroid cancer.

Purification of MBP-TPO Fusion Proteins. Because of the evidence of serologic responses to TPO in thyroid cancer patients, an evaluation of cellular immune responses to TPO in these patients was performed. It was postulated that some form of cellular immune response was necessary to generate the anti-TPO antibodies, and in autoimmune thyroiditis immune reactivity to TPO is associated with cellular cytotoxic responses to thyroid cells. Since cellular immunity to thyroid cells was likely to be a crucial factor in the immune response to cancer, it seemed an important factor to evaluate in cancer patients. Unfortunately, the immunoreactive TPO fusion proteins isolated from the amylose affinity column demonstrated a broad range of molecular weight fusion proteins, as shown in Figure 10, and were not of a purity suitable for use in cellular studies. This made defining the precise location of T cell epitopes difficult, and the problem was further compounded because the material appeared to contain a predominance of maltose binding protein sequence. In an attempt to resolve this problem, the pMALcRI plasmid was transfected into in lon and rpoH am165 deficient *E. coli* strain (data not shown) and fusion proteins were produced in these bacteria. This procedure still yielded fusion proteins with molecular weights below the expected size for the full-length fusion translation product. This again suggested that these smaller products were not the result of protease digestion. It appeared that since the shorter-length translation fragments bound and were affinity purified on the amylose column, these smaller fusion proteins must be C-terminal truncations since all but 10 amino acids of the N-terminal of MBP are necessary for binding to maltose (Personal communication, New England Biolabs). Thus, the largest

fusion proteins having a molecular weight equal to the combination of the full TPO sequence and MBP should contain the whole TPO sequence.

An attempt was made to isolate the full-length translation products by molecular weight separation. A number of techniques were evaluated for this, with HPLC proving to be the only technique that provided adequate separation of the different molecular weight MBP-TPO fragments. Unfortunately, the amount of material isolated by this method was minimal and insufficient for immunologic studies. A technique that employs preparative SDS-PAGE (Prep Cell, BioRad, Richmond CA) to isolate the different molecular weight MBP-TPO (AA 633-933) fusion proteins within $\pm$ 2% of molecular weight was employed. As shown in Figure 11, the large number of different molecular weight fusion proteins isolated using amylose affinity columns (MAC-labelled lane) were subsequently separated by molecular weight using preparative SDS-PAGE. The full length translation product (FLP labelled lane) was isolated using this technique and is shown next to a number of other fractions isolated from the mixture (lanes A-L). This technique proved highly effective, yielding hundreds of micrograms of purified material that was strongly recognized by sera from patients with Hashimoto's disease, and it allowed the purification of a full-length fusion protein from whole bacterial lysate in twosteps. The final material was found to be free of other proteins and was also negative for endotoxin on limulus assay. This purified material was appropriate for use in T cell studies.

Cellular Immunity to MBP-TPO Fusion Proteins. The response of peripheral blood mononuclear cells to stimulation with TPO fusion protein (MBP-TPO AA 456-933), control fusion protein (MBP-LacZα), media and PHA controls is shown in Figure 12. Cells were incubated with the antigens for 96 hours, then pulsed with tritated thymidine and harvested 24 hours later. Specific proliferation was observed only to the 10 μg/ml concentration of the MBP-TPO fusion. Lower concentrations of antigen showed no effect. MBP-TPO (AA 456-933) fusion proteins isolate from the Prep Cell were specifically recognized by peripheral blood mononuclear cells from patients with Hashimoto's thyroiditis, suggesting the presence of epitopes recognized by human T cells in the TPO sequence. The demonstration of this reactivity in thyroiditis patients prompted further examination of cellular responses in thyroid cancer patients.

In an attempt to evaluate cellular reactivity to the TPO fusion proteins, Prep Cell purified MBP fusion proteins representing the TPO fragments AA 456-633 and AA 633-933 were used to stimulate peripheral blood mononuclear cells from cancer patients. Prep Cell purified maltose binding protein-LacZα fusion proteins and native

human TPO (isolated from thyroid microsomes using the Prep Cell) were used as controls. The cells were incubated for 96 hours with different concentrations of purified thyroid antigens, and were then pulsed with thymidine and harvested 24 hours later. As shown in Figures 13A and 13B, specific proliferation was seen in response to one of the TPO fragments (AA 456-633), but not to the other fragment (AA 633-933), control MBP fusion proteins (LacZα) or native TPO. A third patient with follicular thyroid cancer was also evaluated, and showed a similar pattern although the total counts ranged from 200-600 total CPM. In two of the patients evaluated, specific proliferation in response to the AA 456-633 fragment was clearly demonstrated. This proliferation was not only greater than the MBP control, but also greater than that seen to native TPO. The reason for this is not clear, as the gravimetric concentrations of the proteins were equivalent and the molar concentrations of the different proteins were only 1.5 fold different. It is possible that the native TPO, which is heavily glycosylated, requires more processing by antigen-presenting cells, thus making it less effective than the recombinant protein in stimulating antigen-specific T cell proliferation. However, the stimulation seen with the TPO fusion protein was greater than that observed with the MBP-LacZα fusion protein, indicating that the stimulation was not due to MBP amino acid sequences. These results indicated that there was pre-existing cellular immunity to TPO in the cancer patients and this immunity could be stimulated by recombinant protein fragments of TPO.

With the lymphocyte proliferation studies providing evidence of cellular immunity to the TPO proteins in cancer patients, studies were performed to determine if there was also evidence of cytotoxic activity related to this cellular immunity against TPO. The most likely source of T cells with this activity would be the lymphocytes infiltrating the thyroid cancer. The thyroidectomy specimen from one of the patients demonstrating proliferative activity to the synthetic TPO fragment in his peripheral blood lymphocytes was obtained. The patient underwent a thyroidectomy and was found to have papillary cancer with a localized mononuclear cell infiltrate surrounding his cancer. The lymphocytes from this thyroid gland were isolated, and the T cells were expanded by two different techniques: culture of the cells for two weeks with either supernatant fluid of lymphokine activated killer (LAK) cells or with IL2 alone. These expanded lymphocytes were then incubated with antigen alone or in combination with irradiated syngeneic antigen presenting cells (feeder cells) for 12 hours, and TNF production was measured 18 hours after the addition of antigen using a bioassay described in Flick, D.A. et al., *J. Immunol. Meth.* 68:167-175 (1984). As the

LAK supernatant expanded, T cells produced TNF in a non-specific manner, primarily in response to the syngeneic antigen presenting cells. In contrast, the IL2 stimulated cells showed TNF production only in response to the TPO fragments in the presence of antigen presenting cells and were not reactive with autologous mononuclear cells or other non-TPO fusion proteins as shown in Figure 14. This result indicated that there were lymphocytes infiltrating the thyroid that could specifically recognize TPO fragments in the presence of antigen presenting cells and respond by secreting TNF. In addition, the specificity of this response could be maintained while expanding the cells under appropriate culture conditions.

Induction of de novo Immune Response to Native TPO by TPO Fusion Proteins. With the finding that the fusion protein TPO fragments were immunologically recognized by the thyroid cancer patients, the characterization of the antigenicity of these constructs was expanded to determine whether the recombinant TPO fusion proteins could elicit an immunologic response to the native TPO molecule. This attribute would be essential if these constructs were to be used as immunizing agents in cancer immunotherapy. To examine the immunogenicity of the TPO constructs in animal models, three Balb-C mice received intraperitoneal immunizations with 2 mg/ml of Prep Cell purified MBP-TPO (AA 633-933) in Freund's adjuvant in an effort to produce antibodies against TPO. Serologic immune responses to the fusion protein and native TPO were evaluated in these animals. In the Western blots of Figure 15, specific reactivity with all of the fusion proteins (lanes A and B), as well as the MBP (lane D) was shown. Most importantly, specific reactivity with TPO (lane C, 100-105 kDa band) was seen. The protein stain of the gel is shown at the right of the blot for comparison. As shown in Figure 15, all three mice developed high titer, specific antibody to native TPO, clearly indicating the development of a serologic immune response to the fusion protein that cross-reacted with native TPO. This indicated that the TPO sequences in the fusion proteins induce antibodies reactive with the native TPO.

The finding that these mice developed strong serologic reactivity to native TPO led to evaluation of potential cellular responses to TPO in these animals. The spleen of one of the immunized mice was harvested for a hybridoma fusion and a portion of the spleen preparation was used for cellular studies. The mouse splenocytes were immunized with Prep Cell purified MBP-TPO (AA 633-933) in complete Freund's adjuvant. This animal was documented to have developed specific antibody to native TPO through this immunization. See Figure 15, Mouse 2. Proliferation assays were

conducted using the purified fusion proteins, MBP and native TPO, as shown in Figure 16. As might be expected in an animal recently immunized with Freund's adjuvant, there was high background (spontaneous) proliferation of the cells. This made appraisal of the proliferation to the antigens difficult. However, the results suggest that the mouse lymphocytes proliferated in response to native TPO, although not significantly when compared to background. Further studies are currently being performed with several technical modifications: the animals are immunized with the fusion proteins in the absence of Freund's adjuvant and the assays are being performed in serum free media. The cellular immune response to native TPO is then measured.

SPECIFIC EXAMPLE III - IMMUNE RESPONSE TO TPO

Further evaluation of anti-TPO responses in cancer patients provides a more definitive understanding of which immune characteristics determine the extent of a patient's response to immune stimulation with TPO and assists in screening potential recipients for TPO-based cancer vaccine.

Immunoassay Techniques for the Determination of Antibody Responses to TPO in Cancer Patients. Serologic immunity to TPO is quantified by a number of different antibody assays in order to obtain a clear understanding of degree and specificity of this response. Traditional anti-microsomal antibodies are determined by commercial agglutination assay (Ames Diagnostics). Antibodies to human TPO are determined by ELISA using Prep-Cell-purified antigen with techniques described in Weetman, A.P. et al., *Clin. Chim. Acta.* 138(3):237-244 (1984). Antibody to the localized epitopes described in Specific Example I is assayed through Western blots of MBP fusion proteins containing specific TPO amino acid sequences (see Maastricht, J. et al., *J. Clin. Endocrinol. Metab.*, accepted for publication 1992) or through ELISA using Prep Cell-purified MBP-TPO fusion proteins. The latter assay involves coating 96-well plates with 1 μg/ml of fusion protein, then blocking the plates with BSA. The patient sera is run in quadruplicate, 1:2 serial dilutions (from 1:20) in phosphate buffered saline containing an excess of purified MBP, and the end-point titer determined. A control positive and negative sera is run on each plate to standardize the inter-plate variability. Antibodies to thyroglobulin are determined using a commercial agglutination assay (Ames Diagnostics) as a measure of overall serologic immune activity to thyroid antigens.

Examination of Cellular Immune Responses to TPO in Thyroid Cancer Patients. In order to determine if cellular immune responses to TPO are present in

the cancer patients being evaluated, several techniques are utilized. Proliferation in response to specific antigen is performed as previously described in Fisfalen, M.E. et al., *J. Clin. Endocrinol. Metab.* 66(4):776-784 (1988) using Prep Cell-purified native TPO isolated from thyroid microsomal fractions. See Yokoyama, N. et al., *J. Clin. Endocrinol. Metab.* 70:758-765 (1990). In addition, because of the divergent results between native and recombinant TPO in preliminary studies, at least three different MBP-TPO fusion proteins, MBP-TPO (AA 1-400), MBP-TPO (AA 376-631) and MBP-TPO (AA 456-933) are produced that will cover the entire amino acid sequence of TPO (with amino acid overlap to ensure all potential epitopes are identified). These fusion proteins are purified using the prep cell and used to stimulate peripheral blood mononuclear cells (PBMC) from the patients in a manner identical to the native TPO. Control stimulations are performed with MBP-LacZα fusion proteins. If proliferation with MBP is identified, the patients are assayed using recombinant TPO fragments separated from MBP after cleavage with Factor Xα.

Cytotoxic immunity for thyroid cells is measured with a chromium release assay using autologous thyroid cells as targets. These normal cells are isolated from normal, peri-tumor tissue in thyroidectomy specimens and grown in primary culture before labelling as described in Sack, J. et al., *Cancer* 59(11):1914-1917 (1987). TPO-specific cytotoxicity is determined using several assays. Specific production of TNF in response to antigen is performed as outlined in Flick, D.A. et al., *J. Immunol. Meth.* 68:167-175 (1984), with TNF production measured at several time points after the addition of antigen. Specific cytotoxicity for TPO-targets cannot be measured using thyroid cells because of the presence of other thyroid antigens. An alternate method uses autologous EB-lymphoblastoid cells transfected with the TPO gene and selected for TPO expression. The production of these targets and the cytotoxicity assay is described under the section of this Specific Example entitled *Induction and Characterization of CD8$^+$T Cells Specific for TPO.*

Determination of TPO Expression on Thyroid Cancer. TPO expression is evaluated using immunohistochemical staining techniques discussed in DeMicco, C. et al., *Cancer* 67:3036-3041 (1991). Antibodies used for this detection involve monoclonal antibodies against several portions of the TPO molecule (see DeMicco, C. et al., *Cancer* 67:3036-3041 (1991); Finke, R. et al., *J. Clin. Endocrinol. Metab.* 71:53-59 (1990); and Hamada, N. et al., *J. Clin. Endocrinol. Metab.* 61(1):120-238 (1985)) as well as hetero-antisera raised against recombinant portions of the TPO molecule (see Figure 15). The tumor is also cultured, the cells harvested and whole

cell lysate prepared and subjected to SDS-PAGE, when possible. See Baker, J.R. Jr. et al., *J. Immunol.* 140(8):2593-2599 (1988). Western blots of these lysates are performed to determine if protein is produced *in vitro* by these cells. If immunoreactive TPO is not detected in the tumor, the thyroid cancer cells are cultured and harvested as described in Rapoport, B. et al., *J. Clin. Endocrinol. Metab.* 58(2):332-338(1984), and RNA is isolated as described in Ledent, C. et al., *Endocrinol.* 129(3):1391-13401(1991). Northern blots of this RNA probed with labelled TPO cDNA (see Ozaki, S. et al., *Cell Immunol.* 185:301-316 (1987)) are used to detect evidence of transcription of the TPO gene, and to determine if the lack of expression is due to a defect in transcription or translation. This may indicate why the tumors have recently lost the capability to produce TPO or if tumors may be producing minimal amounts of TPO protein or protein fragments not detected through the immunohistochemical staining techniques employed.

Induction of CD4/Helper Type Cellular Immunity to TPO. Techniques to induce and amplify cellular immunity to TPO in patients with thyroid cancer should emphasize the induction of TPO-specific immunity in both CD4 and CD8 cellular subsets, as several animal tumor models have shown that the combined activity of both types of immune cells is most effective in mediating regression of established tumors. However, the methods of generating and characterizing TPO-specific immunity in CD4 and CD8 cells is markedly different, as is the interaction of each subset with antigen. Therefore, the two T cell subsets are examined separately.

$CD4^+$ T cells are thought to function by T cell antigen receptor (TCR) recognition of antigenic peptides presented in conjunction with Class II HLA molecules. This triggers the cells to produce lymphokines, such as IL2, IL4, TNF, INFγ and GM-CSF, which stimulate a number of immune functions. These lymphokines normally have minimal inherent cytotoxicity, however they induce and activate direct cytotoxicity by $CD8^+$ T cells, LAK cells and macrophages. In addition, it is possible that released lymphokines may cause autologous stimulation that results in $CD4^+$ T cells evolving into active $CD8^+$ cytotoxic T lymphocytes (CTL). Thus, $CD4^+$ cell cytotoxicity may occur through both direct and indirect means.

Induction and Expansion of TPO-specific $CD4^+$ T Cells. The induction of $CD4^+$ T cells specific for TPO is conducted on PBMC from patients with thyroid cancer. One of the advantages of studies involving $CD4^+$ T cells is the relative ease (compared to $CD8^+$ T cells) that these cells can be grown in culture. Because antigen-presenting cells will take up exogenous antigen, process and present it in

conjunction with Class I MHC, soluble antigen and peripheral blood mononuclear cells are essentially the only requirements to induce growth and stimulation of $CD4^+$ T cells. However, there are many variations in this process, especially in the composition of the antigen and the type of antigen-presenting cell, that can markedly influence not only the state of activation but the type of lymphokine produced. These variables are fully evaluated in the following studies.

TPO-specific $CD4^+$ T cells are developed from PBMC which are purified from the peripheral blood of patients using Ficoll-Hypaque gradients. To produce the antigen-specific cells, approximately 1×10^6 cells/ml are incubated with purified forms of TPO antigen. These antigens include purified human TPO isolated from thyroid microsomes by the Prep Cell and recombinant TPO fragments coupled to MBP. It has previously been determined with cloning studies in autoimmune thyroiditis, that between 0.1 to 100 ng/ml of antigen should be incubated with the mononuclear cells as an optimal concentration for stimulation. See MacKenzie, W.A. et al., *J. Clin. Encrinol. Metab.* 64(4):818-824 (1987). Antigen stimulation is done for five days in RPMI media supplemented with 10% pooled normal human serum, or in serum-free media (ABC media, Pan Data Systems, Gaithersberg MD). After the five days of stimulation, the reactive lymphoblasts are cultured for an additional seven days in media containing ten units/ml of recombinant IL2 (Genzyme, Boston MA) or 10% PHA supernatant of peripheral blood cells prepared as described in Higgs, J.B. et. al., *J. Immunol.* 140(11):3758-3765 (1988). After approximately seven days in IL2, the lymphoblasts are collected, washed and again stimulated with antigen for three days in the presence of irradiated, autologous peripheral blood mononuclear cells in IL2 free media. These alternating antigen/IL2 cycles are repeated approximately four to six times, with antigen stimulation always followed by IL2 stimulation.

Proliferative Response of TPO-specific T Cells. After these cycles, the cells are evaluated for antigen specificity through proliferation assays using purified antigen and irradiated antigen-presenting cells. Proliferative responses are determined using thymidine incorporation as described previously in Holoshitz, J. et al., *J. Clin. Invest.* 73(1):211-215 (1984). Approximately 15×10^4 cultured T cells and approximately 7.5 $\times 10^4$ irradiated autologous PBMC are incubated together per well, with or without the antigen for approximately 72 hours. Cultures are then pulsed with ^{3}(H) thymidine and harvested as described in Holoshitz, *supra*. Proliferated responses are assessed by comparing CPM obtained with the antigen to CPM obtained without the antigen, and with PHA-stimulated cells serving as a positive control. A stimulation index of

approximately three times background is usually considered positive. Stimulation indices of TPO antigen versus control antigen (such as tetanus toxoid) is compared to determine the enhancement of specific TPO responses achieved through the sequential stimulation cycles.

Production of TPO-specific CD4$^+$ T Cell Clones. TPO responsive cells are also cloned using previously described limiting dilution techniques. See MacKenzie, W.A. et al., *J. Clin. Endocrinol. Metab.* 64(4):818-824 (1987) and Goronzy, J. et al., *Meth. Enzmol.* 150:333-341 (1987). This technique involves Percoll density gradient purification of the viable lymphoblasts which are then re-suspended to a concentration of approximately 5 cells per 100/ml. These cells are then plated onto flat bottom 96-well microtiter plates (Costar) at approximately 100μl per well, over feeder cells consisting of autologous, irradiated EB transformed B cells or PBMC. The autoantigen are reintroduced at a concentration ranging from approximately 1 ng/ml to 1 μg/ml. The cloned cells are then examined for proliferating cells under phase-contrast microscopy. Those cells which appear to be responding are expanded through weekly repeated stimulations with feeder cells and antigen, and are then expanded to large populations with IL2.

Phenotyping of T Cells and T Cell Clones. The surface phenotype of the successfully propagated, TPO-specific cells and clones is determined using cytofluorographic analysis as described in Lanier, L.L. et al., *Nature* 324(6094):268-270 (1986). Antibodies include: anti-CD5 (Becton-Dickinson), OKT11 (anti-CD2, Ortho-Diagnostics), anti-leu4 (anti-CD3, Becton-Dickinson), anti-leu3α (anti-CD4, Becton-Dickinson), anti-leu2α (anti-CD8, Becton-Dickinson), WT31 (anti-α β T cell receptor, Becton-Dickinson) anti-DR (Becton-Dickinson), anti-IL2 receptor (Becton-Dickinson), TCR δ 1 (anti-TCR δ chain, T Cell Sciences) and W6-32 (anti-HLA A, B & C, Accurate Scientific). Two color staining is performed and the clones are judged positive only if greater than 90% of the cells express an antigen as compared to isotope control monoclonal antibodies labeled with the same fluorescent dye.

Localizing CD4$^+$ T Cell Epitopes in TPO. It may be important to identify the precise epitopes involved in the induction of CD4$^+$ T cell immunity to TPO. These small peptide fragments are then coupled to a number of adjuvants in order to increase their immunogenicity and augment the immune response to native TPO. The localization of these epitopes involves the production of recombinant TPO fragments. This work is conducted in a manner similar to that described in Specific Example I and Maastricht, J. et al., *J. Clin. Endocrinol. Metab.* 75:121-126 (1992). Essentially,

fragments of the cDNA for TPO are produced through digestion with specific endonucleases and Polymerase Chain Reaction (PCR). These fragments are ligated, in correct frame for translation, into pMALcRI. The plasmids created are used to transform XL-1 *E. coli*, and lysates of these cells are prepared. The MBP-TPO fusion proteins are isolated from lysate by amylose affinity chromatography and preparative SDS-PAGE. These fragments are used in proliferation assays to identify which TPO fragments contain the amino acid sequences recognized by the $CD4^{+}$ T cells.

Once the epitope has been localized to approximately 20 amino acids using the TPO fragment fusion proteins, synthetic peptides are used to map the exact T cell recognition sequence. Because T cells normally recognize peptides of 6-12 amino acids in length, 12 amino acid peptides which overlap by 6 amino acids are constructed which correspond to suspected epitopes. The synthetic peptides are tested to determine if they induce proliferation in the TPO-specific T cell and clones. Those peptides with activity are subdivided into smaller peptides, each containing a fraction of the 12 amino acid sequence, to indicate the exact recognition site.

Alternative Studies. It is possible that despite favorable preliminary data, the recombinant proteins may not be entirely effective in inducing a TPO-specific CD4 immune response, or may only produce a response in a minority of patients. Several techniques are utilized to overcome this problem. The synthetic TPO fragments are cleaved from MBP and coupled to more potent adjuvants, such as KLH. See Bruderer, U. et al., *Immunol.* 64(3):385-390 (1988). The concentrations of these fragments may need to be varied, or several different fragments of the TPO molecule may need to be mixed together to provide adequate stimulation of many different anti-TPO T cell clones all recognizing different epitopes. Different types of antigen presenting cells may also be important in the induction of CD4 responses. Dendritic cells are more effective in presenting antigen than normal macrophages and are isolated from peripheral blood using monoclonal antibodies and flow cytometry, or preferably with monoclonal antibody-coated magnetic beads (Dynal). Normal, autologous thyroid cells may be useful in this context, especially if Class II antigens are induced with γ-interferon. In addition, it is possible that EB-transformed lymphoblastoid cells are a superior method for stimulating CD4 cells. These options are exercised if initial attempts to induce TPO-specific CD4 cells are not successful.

A major factor guiding the *in vitro* production of CD4 immunity may be the natural development of anti-TPO immunity in cancer patients *in vivo*. In this regard, studies previously discussed in this Specific Example, should serve as an indication

of the degree of $CD4^+$ T cell sensitization and its relation to tumor TPO expression. Once the prevalence of this immunity is established, through the evaluation of large numbers of patients, subsequent studies focus on the specific conditions which affect the development of TPO-specific $CD4^+$ T cell responses *in vivo*. In relation to this, several clinical factors are closely examined: tumor histology, stage, evidence of metastasis, nutritional status and prior treatments. It is possible that the anti-TPO response is maximal early in the course of the disease, and becomes attenuated by the presence of increased tumor burden, metastasis and chemotherapy. To maximize the yield of TPO-specific precursor $CD4^+$ T cells for *in vitro* manipulation or expansion, it is important to understand the relationship between the tumor and anti-TPO responses and address whether endogenous TPO-specific $CD4^+$ T cells immune responses modulate the biology of autologous tumors *in vivo*.

Induction and Characterization of $CD8^+$ T Cells Specific for TPO. Cytotoxicity of tumor cells by Class I MHC-restricted $CD8^+$ cells has been demonstrated in a number of tumor models. See Mitsuya, H. et al., *J. Exp. Med.* 158:(3):994-999 (1983) and Anichini, A. et al., *J. Immunol.* 142(10):3692-3701 (1989). While technical problems are encountered in generating these cells, they offer the possibility of direct tumor lysis using restriction elements (Class I MHC antigens) and target antigens already present in the tumor cells. Because Class I antigens preferentially express endogenously-derived peptides to the exclusion of exogenous ones as discussed in Germain, R.N., *Nature* 322 (6081):687-689 (1986); Berzofsky, J.A. et al., *Immunol. Rev.* 106:5-31 (1988); and Grey, H.M. et al., *Sci. Am.* 261(5):56-64 (1989), they will tightly restrict the response to the desired target cells. Because this process is so markedly different from $CD4^+$ T cell recognition of antigen, this experiment is devoted exclusively to an examination of $CD8^+$ T cell recognition of TPO-bearing cells and the function of these cells in mediating tumor lysis.

Preliminary studies have demonstrated peripheral blood and intrathyroidal lymphocytes from cancer patients to be sensitized to TPO *in vivo*. However, to detect antigen-specific CTL, it is usually necessary to re-stimulate cells *in vitro* to induce functional CTL. In general, these studies involve the isolation of $CD8^+$ T cells from thyroid cancer patients, their stimulation with various types of cells bearing different forms of TPO and a determination of the CTL activity induced by these stimulations. A major consideration is the supplementation of the growth of the CD8 cells in culture with exogenous cytokines. This is essential because $CD8^+$ T cells do not produce adequate quantities of autocrine factors to support their own growth. In this regard,

different combinations of supernatant fluid from activated lymphocytes, irradiated feeder cells and specific lymphokines (such as low dose, $\leq$ 10 μIU, recombinant IL2) is added to support the growth of these cells. Techniques for inducing large numbers of TPO antigen-specific cytotoxic T lymphocytes, either *in vitro* or *in vivo* are thus developed.

Induction of TPO-specific CD8$^+$ CTL in vitro by TPO-bearing Thyroid Cancer Cells. A major hypothesis of this study is that thyroid cancer cells expressing TPO protein can be lysed by CTL-specific for tumor-associated TPO. The ability of these same cells to elicit this type of response prompts the investigation of the induction of a TPO-specific CTL response. Tumor cells are isolated from thyroidectomy specimens through mechanical isolation of the tumor followed by enzymatic digestion as described in Rapoport, B. et al., *Metabolism* 31:1159-1167 (1982). These cells are cultured for at least 6-8 passages *in vitro*, and the tumor cells bearing TPO are isolated with FACS or magnetic beads and anti-TPO. Approximately 1 x 10^5 tumor cells are co-cultured for 4-6 days with 4 x 10^5 CD8$^+$ cells in the presence of various concentrations of IL2 and feeder cells. The CD8$^+$ cells are isolated from PBMC using magnetic beads (Dynal) with either anti-CD8 (for positive selection) or anti-CD4 (for negative selection). The growth of these cells is monitored and surface markers, such as LFA-1, CD2, and other molecules involved in adhesion for cytolysis are identified through FACS. Other combinations of cytokines may include IL4 in place of IL2 to prevent the potential induction of non-specific LAK cells (see Kawakami, Y. et al., *Exp. Med.* 168(6):2183-2191 (1988)), or the addition of such factors as INFγ, IL-1, TNFα or supernatant fluid from mitogen (PHA) stimulated lymphocytes, if the initial conditions are not adequate to induce TPO-specific CTL activity.

Induction of TPO-specific CTL by Autologous Non-tumor Cells Incubated with Synthetic TPO Peptides. Although thyroid cancer cells may be very effectively lysed by CD8$^+$ CTL, the tumor cells may not be appropriate cells to use to stimulate the generation of these cells. Various cytokines elaborated by tumors, such as TGFβ, can inhibit the differentiation of CTL. See Wrann, M. et al., *EMBO J.* 616:1633-1636 (1987) and Ranges, G.E. et al., *J. Exp. Med.* 166(4):991-998 (1987). The presence of extraneous, non-tumor specific antigens presented aberrantly by the tumor cells may obscure the detection of an anti-TPO response. Some of the determinants from these antigens may preferentially bind to Class I antigens and block binding of TPO peptides, thereby preventing the generation of an anti-TPO response. If specific,

synthetic TPO fragments are not generated, the difficulties with using tumor cells are overcome by using normal cells that do not endogenously express TPO. Under certain circumstances extra-cellular soluble peptides can bind to Class I molecules as discussed in Townsend, A.R. et al., *Cell* 44(6):959-968 (1986). While the Class I molecules are normally occupied by cell peptides (see Bjorkman, P.J. et al., *Nature* 329(6139):506-512 (1987)), some alterations in culture conditions, such as culture at 27°C, results in the expression of Class I antigens without endogenous peptides. See Ljunggren, H.G. et al., *Nature* 346(6283):476-480 (1990) and Shumacher, T.N. et al., *Cell* 62(3):563-567 (1990). The addition of TPO peptides to these cells, especially accompanied by β-2-microglobulin as discussed in Kozlowski, S. et al., *Nature* 349(6304):74-77 (1991), may allow adequate amounts of complexes to form so that these cells can then present TPO peptides to $CD8^+$ T cells. Possible cell lines used for this include autologous fibroblasts, grown from the surgical thyroidectomy specimen, or autologous EB-transformed lymphocytes. Both of these cells express Class I molecules, and since they are autologous, would share the same Class I allotype. These cells are different in that the lymphoblastoid cells can constitutively express Class I MHC while the fibroblasts do not. The effect of the differences in expression of these molecules or other differences in these cells on CTL generation is unknown, and is one parameter that can be monitored in these studies to provide important general insights into the induction of antigen-specific CTL.

The TPO peptides used to coat these cells are produced from the MBP-TPO (AA 456-933) fusion proteins. The TPO-specific areas are isolated by cleavage with Factor Xα, and then are manipulated by heat denaturation, detergent or protease treatment before being added to the culture to yield peptides of TPO that will bind to the Class I MHC antigens. Truncated versions of these peptides, produced from fusion proteins like MBP-TPO 456-631, are also employed to better define the specific $CD8^+$ T cell epitopes, and eventually yield a more specific and intense CTL response. If the specific epitopes are determined in this manner, synthetic peptides are then used to replace the fusion-protein derived TPO sequences.

Induction of TPO-specific CTL Activity by Cells Transformed with Viral Vectors Containing TPO cDNA. One of the advantages of having the full-length cDNA for TPO is that it can be used with a number of eukaryotic vectors to express the TPO protein in cells where this antigen is not normally found. The TPO cDNA may be placed into several vectors as described in Wilson, J.M. et al., *Science* 248(4961):1413-1416 (1990) and Shigekawa, K. et al., *Biotechniques* 6:742-751 (1988),

for expression in several types of autologous cells from the patients and produce cells with endogenous expression of TPO. After transformation, these cells are selected with G418, the common name referencing geneticin, as sold by Sigma Chemical, and those cells expressing large quantities are identified and isolated using FACS and monoclonal anti-TPO. This results in non-thyroid cells, such as fibroblasts, over-expressing TPO, and may provide important cells for the induction of CTL. EB-lymphoblastoid cells expressing TPO have both Class I and Class II MHC antigens, and can also be used to induce both $CD4^+$ and $CD8^+$ anti-TPO immune responses. These cells can also provide targets to identify TPO-specific CTL activity, as control cells transformed with vectors expressing irrelevant or no protein can be easily generated. Using these cells to induce CTL activity provides a unique approach resulting from a cloned and expressible tumor antigen.

Determination of Cytotoxicity against TPO Target Cells. Cytotoxicity against *in vitro* cultured thyroid cells, thyroid cancer cells or TPO gene-transfected target cells is assessed using ^{51}Cr release assay as described in Sack, J. et al., *J. Clin. Endocrinol. Metab.* 62(5):1059-1064 (1986). Thus cells are incubated with ^{51}Cr for labeling. Excess chromium is washed off and these labelled target cells are mixed with cloned $CD8^+$ T cells at various target ratios for 4 hours at 37°C, in a humidified atmosphere of 5% CO_2. Supernatant fluids are harvested and counted in a gamma counter, each effector/target ratio determined in triplicate and the data presented as specific ^{51}Cr release measured as the experimental release minus spontaneous background over the total counts present minus spontaneous background x 100, thereby giving the ratio of reactivity. The total counts releasable is determined by mixing the cells in 1% Triton x100. Spontaneous release is determined by measuring the ^{51}Cr release of the target cells cultured in media alone without cytotoxic cells. To document the MHC Class I restriction of CTL by the $CD8^+$ T cell clones, blocking experiments with monoclonal antibodies against Class I or Class II antigens (as controls) are also conducted. Results are collected and analyzed to determine the specific requirements for Class I antigens necessary for TPO antigen-specific CTL.

Evaluation of Molecular Elements Involved in CD8 Recognition and Binding to Tumor Cells. Determining the exact epitopes in TPO that are recognized by the $CD8^+$ T cells is of primary importance. While it might be possible to generate anti-TPO activity *in vitro* with synthetic TPO, the CD8 target epitopes must be present on the cancer cells to make cytotoxic immunity effective. Using the recombinant fusion proteins and synthetic peptides described previously will allow the identification of the

specific portions of the TPO molecule recognized by the CD8 cells, and allow the determination of whether each tumor express a particular TPO epitope.

It is possible that the CTL lysis may be restricted to certain MHC Class I allotypes, either because of the inability to bind TPO peptides or the inability of the MHC-TPO peptide complex to be recognized by CD8 T cell antigen receptors. If this appears to be the case, cells from patients who appear able to present TPO peptides are tissue typed and the specific allotypes necessary to present these antigens are identified. This allows screening of patients who are most likely to respond to therapy with this antigen.

Effect of TPO on Lymphokine Production by T Cell Clones. Because of the important function lymphokines are believed to play in the induction of cytotoxic immunity to cancer cells, it is important to determine whether the T cells and T cell clones produce lymphokines in response to specific stimulation with antigen. Commercial immunoassays for TSH, IL2, IL4, IL5, IL6, TNF and interferon γ are run on antigen-stimulated T cell clone supernatant fluid to determine whether or not these lymphokines are produced by the clones. Bioassays for lymphokines with more generalized actions that may mediate direct cytotoxic actions, such as TNF discussed in Flick, D.A. et al., *J. Immunol. Meth.* 68:167-175 (1984), and IL6 discussed in Ida, N. et al., *J. Immunol. Methods* 133(2):279-284 (1990), are also performed.

In some situations, lymphokines that are membrane bound or are delivered in local interactions between CTL and target cells may play an important role in cytotoxicity but may not be detectable in supernatant fluid. To attempt to identify these factors, the activation of transcription of the genes for IL2, IL3, IL4, IL5, IL6, interferon γ and TNF in the T cell and clones is determined using PCR techniques. The PCR studies involve the cells being isolated and lysed with 100 µl of GITC (guanidine-isothiocyanate) solution with the resulting fluid loaded onto a 100 µl cesium chloride gradient in ultracentrifuged tubes. These tubes are spun at 80,000 RPM for 2 hours at 20°C, after which the supernatant fluid is aspirated and the pellet combined with 100 µl of DEPC (*i.e.* diethylpyrocarbonate) treated water to resuspend the RNA. Potassium acetate (10 µl) is added to the tubes and the RNA is cold ethanol precipitated. The precipitate is then washed with 80% ethanol and the samples are air-dried. AMv reverse transcriptase will be used to produce first strand DNA from approximately 100 ng of RNA in a 50 µl reaction at 37° for 1 hour as described in Okayama, H. et al., *Mol. Cell. Biol.* 2(2):161-170 (1982). Ten µl of first strand cDNA is mixed with 80 µl of PCR mix (containing dNTP's mix, 200mM and Taq polymerase,

2.5 units per assay tube, Perkin Elmer/Cetus). Five µl of primers for each lymphokine is added to each tube to give a final primer concentration of 1 µM. The mixture is then subjected to PCR amplification using the Perkin-Elmer thermocycler. An ethidium bromide stained 2% agarose gel is used to separate the PCR fragments. Inclusion of a sample from a cell line known to transcribe the assayed lymphokine (*e.g.*, Jurkat cells for IL2) is used as a positive control for each experiment. For a negative control, "mock" reverse transcription reactions are performed on RNA by omitting the reverse transcriptase enzyme and subjecting this mixture to PCR. In this way, the activation of lymphokine transcription induced by antigen is determined even in the very slight lymphokine release from the cells (on the order of µM).

Effect of Lymphokines on Thyroid Cancer Cell Function. Assays of thyroid cell function using thyroid cancer cells in primary culture are carried out whenever adequate numbers of cells are obtained from surgical specimens. The cancer cells are cultured with supernatant fluids from $CD4^+$ and $CD8^+$ T cells and T cell clones. Determinations of the effect of the lymphokines include the quantitation of cAMP produced by the cancer cells as discussed in Vitti, P. et al., *J. Clin. Endocrinol. Metab.* 57 (4):782-791 (1983), and the ability to suppress thyroid cancer cell growth as measured by the induction of DNA synthesis in the measurement of ^3H thymidine incorporation as described in Valente, W.A. et al., *N. Engl. J. Med.* 309 (17):1028-1034 (1983). In addition, the ability of the T cell supernatant lymphokines to suppress cancer cell production of immune-blocking factors, such as TGFβ (see Lucas, C. et al., *Methods Enzymol.* 198:303-316 (1991)) and thyroglobulin (see Feldman, A. et al., *Clin. Endocrinol. (Oxf.)* 25(1):45-53 (1986)), is also assessed. Several lymphokines and cytokines have been reported to have effects on thyroid cells that may either potentiate or suppress cytotoxic actions. IL6 has been reported to suppress TPO expression in normal thyroid cells in Tominaga,Y. et al., *Acta Endocrinol. (Copenh.)* 124 (3):290-294 (1991), and this would be detrimental in TPO-directed cytotoxic therapy if true in cancer cells. Thus, the effects of these cytokines on thyroid cancer cells is important.

Induction of TPO Antigen-Specific TIL Cells from Thyroid Cancer. The induction of TPO-specific immune cells from TIL offers unique potentials and problems. The TIL cells have great potential for use in TPO-specific immunotherapy, as a proportion of the cells were drawn to the tumor site and their numbers expanded due to their reactivity with TPO and other tumor antigens. In addition, there is likely to be preformed CTL cells or other cells capable of mediating tumor lysis. This is supported

by the findings in Specific Example II, where these cells produced TNF in response to stimulation with TPO. Because of this, many of the studies outlined in this Specific Example are performed on the tumor infiltrating lymphocytes harvested from the patients thyroidectomy specimens. However, while the problems outlined in these sections certainly apply to the TIL cells, there are other considerations that make the generation of CTL from the TIL cells unique. Because this is an inflamed area, non-specific LAK and CTL activity are likely the major response, and are separated from the anti-TPO activity. This likely requires the use of different lymphokine for stimulation of antigen-specific immunity, and the cloning of all of these cells at an earlier stage of development. Also, the limited number of cells obtained from these tumors may require greater expansion of the cell populations. Thus, although similar studies are performed on the TIL cells, they require different conditions and techniques to be successful.

The differences in culture conditions for the TIL cells revolves around the initial conditions for growth, *in vitro*. The cells can be cultured as whole, enzymatic digests of thyroid tumors, purposely keeping the cancer cells present as a source of antigen and tissue dendritic cells to present antigen. Alternatively, T cells are isolated from the digested tumor through anti-T cell monoclonal antibody-labelled magnetic beads (Dynal), and cultured either with purified antigen or irradiated tumor cells in the presence of low-dose IL2 (<10 U/ml) and possibly IL4 (to inhibit the LAK activity). The cells expanded by these techniques are then evaluated as outlined above.

In Vivo Models. In addition to Hashimoto patients as human models for TPO immunotherapy, *in vivo* models of thyroid cancer are used to evaluate its efficacy. Implantation of thyroid cancer (thyroidectomy) specimens into SCID/human mice is employed because in comparison to previous models, this model will more closely replicate human disease. Since the immune cells in these thyroid glands should include a percentage of anti-TPO T cells, the immune system of the mouse provides an *in vivo* model to study immune system stimulation. Tumor regression can then be monitored as a measure of efficacy.

SPECIFIC EXAMPLE IV

TPO AUTOANTIBODY RESPONSES IN GRAVES' AND HASHIMOTO'S PATIENTS

Two disorders with markedly different clinical presentations, Graves' disease and Hashimoto's thyroiditis, are both associated with autoantibodies to the microsomal antigen, now identified as the enzyme thyroid peroxidase (TPO).. Libert, F. et al., *EMBO J.* 6:4193-4196 (1987); Portman, L. et al., *J. Clin. Endocrinol. Metab.*

61:1001-3 (1985). Characterizing the immune response to this antigen in patients with these two diseases may help clarify differences in the pathogenesis of the autoimmune thyroid diseases (AITD) and facilitate more accurate diagnosis of thyroid disorders.

Recently published studies have suggested that autoantibodies directed against TPO are heterogeneous in nature. Localized autoantibody binding to a region between amino acids 590 and 675, and between amino acids 700 and 933 have been reported [Libert, F. et al., *EMBO J.* 6:4193-4196 (1987); Libert, F. et al., *J. Clin. Endocrinol. Metab.* 73:857-60 (1991); Nakajima, Y. et al., *Mol. Cell Endocrinol.* 53:15-23 (1987)] and the sizes of the TPO peptide fragments examined in these studies were small enough to suggest that these epitopes were not conformational. Several other reports using purified native TPO have suggested that there may be multiple autoantibody epitopes, some of which are conformational. Doble, N.D. et al., *Immunology* 64:23-29 (1988); Yokoyama, N. et al, *J. Clin. Endocrinol. Metab.* 70:758-65 (1990); Finke, R. et al, *J. Clin. Endocrinol. Metab.* 73:919-21 (1991). Previous work has demonstrated localized autoantibody epitopes in the portion of the human TPO molecule from amino acids 456 to 933, and suggested the presence of multiple binding sites. See Maastricht, J. et al, *J. Clin. Endocrinol. Metab.* 75:121-26 (1992).

In the present invention, the serologic immune response to native thyroid microsomal antigen, denatured and reduced microsomal antigen, and several recombinant peptide fragments of human TPO in patients with Graves' disease and Hashimoto's thyroiditis was characterized. The results were then correlated to measures of clinical disease activity in order to determine if clinical subgroups or associations related to specific types of anti-TPO activity.

Overview. In order to examine the specificity of the autoantibody response to thyroid peroxidase in autoimmune thyroid disease, the reactivity of sera from 45 Hashimoto's and 48 Graves' patients to native thyroid microsome, denatured and reduced human TPO and several recombinant fragments of human TPO corresponding to amino acids 457-933 of the native protein were examined. Both Graves' and Hashimoto's sera bound native, denatured and reduced TPO at significantly greater rates than normal controls, and no differences were noted between the two disorders in binding to these forms of the autoantigen. Binding was also noted to two recombinant fragments of TPO, corresponding to amino acids 513-633 and 633-933 in TPO. The frequency of autoantibodies to the TPO(633-933)region was not significantly different in Hashimoto's vs. Graves' disease patients (58% vs.

65% respectively), and appeared to relate to evidence of glandular inflammation in the Graves' patients (goiter, presence of anti-thyroglobulin antibodies and elevated FT3 levels). In contrast, antibodies to the TPO(AA 513-633) fragment were significantly more common in Hashimoto's vs. Graves' disease patients (53% vs. 23% respectively), and did not correlate with any measure of glandular inflammation. These results identify two specific regions of TPO autoantibody binding and indicate that there are differences in the autoantibody response to TPO in Hashimoto's and Graves' diseases.

Materials and Methods

Patient Sera. Forty-five patients with Hashimoto's disease, forty-eight patients with Graves' disease and twenty-five age and gender-matched normal controls were enrolled in the study. The Hashimoto's patients were diagnosed on the basis of anti-microsomal antibody titers of greater than or equal to 1:400 by agglutination assay in the presence of either goiter or hypothyroidism. The age of the patients ranged from 12 to 77 years. The Graves' patients were diagnosed on the basis of clinical and biochemical evidence of hyperthyroidism (suppressed TSH with elevated T4), diffuse goiter and an increased 24 hour radioiodine uptake. None of the controls had clinical evidence or a past history of thyroid disease. Sera were obtained at the time of enrollment, and frozen at -70° until use.

Construction Of TPO Expression Plasmids. The plasmid pMalTPO(457-933) was developed from pMalcRI (New England Biolabs) and contains (in-frame) the cDNA coding for TPO AA 457-933, as has been previously reported. Maastricht, J. et al, *J. Clin. Endocrinol. Metab.* 75:121-26 (1992). This construct encodes a fusion protein in which the amino terminal portion is maltose binding protein minus its leader sequence, followed first by the sequence IEGRISEF (one letter amino acid code), then the fragment of human TPO. This vector was used to make several new constructs containing fragments of the coding region of the human TPO cDNA from amino acids 457-933. The plasmid pMalTPO(457-633) was developed by digestion of pMalTPO(457-933) with ClaI and XbaI. The linearized plasmid was separated from the excised fragment by agarose gel electrophoresis and the plasmid was ligated to the adapter 5'-CGATTAGGTAGGTAGT (top strand), which recreates the ClaI and XbaI sites and inserts a stop codon after amino acid 633. The plasmid pMalTPO(633-933) was developed by digestion of pMalTPO(AA 457-933) with EcoRI and ClaI with the deletion of the excised fragment followed by Klenow fill-in and religation. The plasmid pMalTPO(AA 513-633) was created by digestion of pMalTPO(AA 457-633) with EcoRI

and SmaI followed by ligation to the adapter 5'-AATTCGACCTGCCC (top strand). The plasmid pMalTPO(AA 457-517) was created by digestion of pMalTPO(AA 457-633) with SmaI and XbaI, isolation of the linearized plasmid and ligation to the oligonucleotide adapter 5'-GGGCTGTAGT (top strand). Each construct was confirmed by restriction digestion and DNA sequencing of the 5' ligation junction.

Expression and Purification of Fusion Proteins. The plasmid constructs were used to transform *E. coli* HB 101, grown, harvested, lysed, and sonicated as previously described in the art. Maastricht, J. et al., *J. Clin. Endocrinol. Metab.* 75:121-26 (1992). The lysate was run over an amylose affinity column to isolate the MBP fusion proteins in accordance with the vendor's protocol, and the fusion proteins were then eluted using column buffer with 10 mM Maltose.

Preparation of Thyroid Microsomal Antigen. Human microsomal antigen was isolated from normal human thyroid tissue obtained under informed consent from thyroidectomy samples as previously reported in the art. Finke, R. et al., *J. Clin. Endocrinol. Metab.* 73:919-21 (1991).

SDS-PAGE and Western Blots. Thyroid microsomal antigen or MBP-TPO fusion proteins were diluted with buffer consisting of 0.125 M Tris pH 6.8 with 4% SDS and 20% glycerol to a concentration of approximately 3 mg/ml. Approximately 500 µl of this solution was loaded into a single preparative well and electrophoresed through an 8% SDS Polyacrylamide gel. For the reducing gels and in all of the MBP-TPO samples, 10% β-mercaptoethanol was added to the sample buffer, and the samples were boiled for 5 minutes before electrophoresis. After electrophoresis, proteins were electro-transferred to nitrocellulose membranes, and these membranes were then blocked with 3% BSA in PBS for two hours at 27°C. Western blots were then performed using a 1:400 dilution of patient sera in PBS with 1% BSA and 0.01% sodium azide. Maastricht, J. et al, *J. Clin. Endocrinol. Metab.* 75:121-26 (1992). For the blots using the fusion protein, the sera were pre-incubated for 3 hours with maltose binding protein-LacZ fusion proteins (0.1 mg/ml) to block any potential reactivity to the maltose binding protein portion of the TPO fusion proteins. Controls included a monoclonal antibodies to thyroid peroxidase and MBP to identify the TPO-specific and MBP-specific bands. Ten patients with high titer anti-nuclear antibodies (ANA>1:6400) randomly selected from the clinical laboratory were also evaluated in the Western blot assays to determine the disease specificity of TPO autoantibodies.

Clinical Measurements. Thyroid function tests (including FT4, FT3, TSH) and thyroglobulin and thyroid microsomal antibody titers were performed on either

identical serum samples used for Western blots or repeat samples drawn within 2 months of the original sample. The thyroid functions were performed in a routine clinical laboratory, while the TSH levels were determined by a sensitive, second generation fluorescence immunoassay. Thyroglobulin and microsomal antibody titers were determined by agglutination assay (Ames Diagnostics, Ames, IA). Goiter size and degree of opthalmopathy were determined by an individual who was unaware of the TPO reactivity of the subjects.

Statistical Analyses. The rates of reactivity to different areas of TPO in the Graves' and Hashimoto's disease patients, and the normal subjects were compared by Fisher's exact test. The continuous variables were compared by using ANOVA.

Results

Reactivity to Denatured and Reduced TPO. As previously reported (Hamada, N. et al, *J. Clin. Invest.* 79:819-25 (1987)), a band of approximately 220 kDa in immunoblots was identified as the non-reduced form of TPO. Under reducing conditions, antibodies against TPO identified three bands ranging from 100 - 105 kDa. Similar patterns of reactivity to these bands was noted in both the Hashimoto's and Graves' patients. As shown in Table 1, the frequency of reactivity of the Hashimoto's and Graves' sera were statistically identical to each other, but markedly greater than that of the control sera.

Table 1

TPO Form	Normal Controls	Graves' Disease	Hashimoto's Disease
Denatured TPO	3/21 (14%)	40/48 (83%) *	34/45 (76%) *
Reduced TPO	1/21 (5%)	23/48 (48%) *	27/45 (60%) *
TPO (513-633)	1/25 (4%)	11/48 (23%) *	24/45 (53%) *‡
TPO (633-933)	2/25 (8%)	31/48 (64%) *	26/45 (58%) *
Both Fragments	0/25 (0%)	9/48 (19%) *	12/45 (27%) *
Either Fragment	2/25 (8%)	33/48 (69%) *	38/45 (84%) *

*P<0.05 vs. Normals ‡P<0.01 vs. Graves'

Autoantibodies against the TPO Fragments. Initial studies failed to demonstrate serologic reactivity with the fragment TPO(AA 457-517) in any of the patient sera, while immunoblots done with either TPO(AA 457-633) or (AA 513-633) showed identical patterns of reactivity. Therefore, the TPO(AA 513-633) region was presumed to contain the autoantibody binding site between amino acids 457-633. Of the 45 Hashimoto's patients studied, 24 (53%) showed reactivity to TPO(AA 513-633), while in the Graves' group, only 11 of the 48 patients (23%) reacted to this region.

Figures 17A and 17B are representative Western blots demonstrating autoantibody reactivity to recombinant TPO (AA 513-633). Sera from Hashimoto's patients (Panel A) and from Graves' patients (Panel B) showed intense reactivity with the fusion protein at 56 kDa (arrow). Control (+) is an anti-MBP antiserum used to identify the location of the fusion protein (arrow). Blots using a control MBP fusion protein indicated no patient binding (data not shown). As shown in Table 1, this difference was highly significant. Only one of the normal controls reacted to TPO(AA 513-633), and the blot showed minimal intensity. In contrast, the frequency of autoantibody reactivity to TPO(AA 633-933) was similar in both the Hashimoto's and Graves' patients. Also, there was no significant difference in reactivity to TPO(AA 513-633) based on whether the patient recognized denatured and reduced TPO, while the TPO(AA 633-933) fragment was recognized at significantly higher rates in patients

reactive to denatured/reduced TPO ($p<0.05$). This suggests an association between autoantibody binding to denatured and reduced TPO and antibodies to the TPO(AA 633-933) region which is not seen with antibodies to the TPO(513-633) region. Of interest, the microsomal antibody titer (as determined by the agglutination assay) did not correlate with binding to either region of TPO or reduced TPO by Western blot in either patient group. Also, none of the 10 control patients with SLE bound to either fragment, suggesting these autoantibodies were disease-specific.

Clinical Correlation with TPO Reactivity. For the Hashimoto's patients, there was no significant correlation between any of the clinical parameters (age, goiter size, anti-microsomal antibody titers and anti-thyroglobulin antibody titers (by agglutination assay) or hypothyroidism) and reactivity to either the recombinant fragments, or the reduced or non-reduced forms of TPO. In the Graves' patients, no statistically significant correlation was seen between age, FT4, ^{131}I uptake, or antimicrosomal antibodies and reactivity to the two regions of TPO or to the reduced and denatured forms of TPO. Correlations were noted between reactivity to reduced TPO and the following parameters in Graves' patients; the presence of anti-thyroglobulin antibody titers ($P=0.04$), with higher levels of FT3 ($P=0.001$) and larger goiters ($P=0.04$). There was no correlation between the presence of ophthalmopathy and reactivity to any portion of TPO. The cDNA for human TPO used for the initial MBP construct was a gracious gift of Dr. Basil Rapoport and has been accorded GenBank Accession Number J02969.

Discussion

These results indicate that the autoantibody response to TPO is truly heterogeneous and that there are at least two localized areas in TPO that are recognized by autoantibodies from a majority of patients with AITD. The recognition of one of the areas, corresponding to amino acids 513-633, was more frequently seen in Hashimoto's disease than Graves' disease. Sera from patients with Hashimoto's disease were equally likely to be reactive to TPO(AA 513-633) and TPO(AA 633-933) while, amongst the Graves' patients, the frequency of reactivity to TPO(AA 633-933) was 3 times higher than to TPO(AA 513-633). This difference did not appear to be the result of differences in the overall serologic response to TPO, as there was no difference between the two disorders in the titer of antibodies to anti-microsomal antigen (by agglutination assay) or reduced and denatured TPO (by Western blot). Thus, the present invention shows that autoantibodies against TPO amino acids 513-

633 are observed more often in Hashimoto's thyroiditis patients as compared to Graves' disease patients.

Specific Example IV of the present invention is thus in direct contrast to the findings of Zanelli, which shows autoantibody binding to all portions of TPO with no difference between Graves' and Hashimoto's patients. (Zanelli, E. et al., *Clin. Exp. Immunol.*87:80-86 (1992)) Note, that the Western blots in Zanelli's studies used sera at a high concentration (1:25 dilution) and did not compare patient binding to normal controls. Therefore, it is difficult to determine if Zanelli (and colleagues) identified autoantibodies specific for autoimmune thyroid disease.

The reason for the association of autoantibodies to the TPO(AA 513-633) with Hashimoto's disease is not clear. It has been speculated that antibodies to localized or sequential autoantigen epitopes are a consequence of denaturation and unfolding of autoantigens. Laver, W.G. et al., *Cell* 61:553-56 (1990). Inflammation of the thyroid gland unique to Hashimoto's disease might result in the denaturation of TPO, which would then result in antibodies to TPO(AA 513-633). However, the reactivity to TPO(AA 633-933) region and to reduced and denatured TPO was not different between Graves' and Hashimoto's patients. In addition, unlike autoantibodies to TPO(AA 633-933), antibodies to the TPO(AA 513-633) fragment did not correlate with serologic reactivity to denatured TPO in the Hashimoto's patients. Therefore, the differences in the rates of reactivity to TPO(AA 513-633) cannot be explained solely as the consequence of an inflammatory process that yields autoantibodies to denatured TPO.

In arguing a pathogenic role for TPO autoantibodies, several studies have shown that anti-microsomal antibodies from patients with Hashimoto's disease have been shown to inhibit the activity of TPO. Okamoto, Y. et al., *J. Clin. Endocrinol Metab.* 68:730-34 (1989). The TPO(AA 513-633) fragment overlaps with a region, amino acids 510 to 567, previously reported to show homology to the heme binding site of the Cytochrome C oxidase enzyme (Libert, F. et al., *EMBO J.* 6:4193-4196 (1987)) and therefore may constitute a functional part of the enzyme. The different reactivity to TPO(AA 513-633) suggests a possible pathophysiologic role for TPO antibodies in Hashimoto's disease and could also explain the variability in the studies examining the effect of autoantibodies on TPO function. However, there appeared to be no significant correlation between reactivity to any form or region of TPO and any clinical characteristic of the Hashimoto's patients. While this could be due to variations in the clinical presentation of Hashimoto's disease and the fact that serum

samples were obtained from the patients at variable times during the course of the disease, it might indicate that TPO autoantibodies do not play a prominent role in the producing clinical signs of AITD (*e.g.*, hypothyroidism or goiter size). To resolve this question, reactivity to this region might be evaluated in a prospective manner in relatives of Hashimoto's patients to see if these autoantibodies presage the development of thyroiditis. In the Graves' patients, a significant correlation was noted between the goiter, anti-thyroglobulin antibody titers and reactivity to both TPO(AA 633-933) and the reduced form of TPO. This may indicate that antibodies to this area of TPO are a marker for glandular inflammation in Graves' disease.

Since Western blot assays are not as sensitive as other immunoassays, and may underestimate the number of reactive patients, there may be more than one epitope in the TPO(AA 513-633) region, only one of which is specific for Hashimoto's disease. In addition, while some of the Graves' patients did react to this area, it is possible that these patients may have concomitant Hashimoto's disease. The coexistence of these diseases is well-documented (Hirota, Y. et al., *J. Clin. Endocrinol. Metab.*62:165-69 (1986)), and the identification of this patient subgroup may be of value because these individuals might develop long-term remissions of their hyperthyroidism due to the presence of the thyroiditis. However, since all of the Graves' patients in this study were treated with thyroid ablation, this hypothesis was not tested.

SPECIFIC EXAMPLE V

EPITOPE MAPPING OF THE TPO 592-613 AUTOANTIBODY BINDING SITE

In order to further localize and understand the specificity of autoantibodies binding to thyroid peroxidase, efforts were undertaken to determine the exact epitopes of TPO autoantibodies directed to the fragment of TPO contained within amino acids. Initially, the AA 517-633 fragment was digested with an additional restriction enzyme to yield a AA 573-633 fragment. This portion of TPO retained the binding activity present in the whole AA 517-633 fragment. However, it became apparent that there were no appropriate restriction enzyme sites to further cut the cDNA. Thus, for fine mapping of this autoantibody binding site, the polymerase chain reaction (PCR) was used.

Figure 18 is a schematic of the fine mapping of the TPO epitope using PCR. Primers were constructed to amplify cDNA segments of approximately 60 nucleotides (20 amino acids) in length. The initial (smallest fragment) was between amino acids 592-613 (20 amino acids from the ends of the 573-633 fragment). The primers were

also prepared with the appropriate restriction enzyme sites at either end for insertion into the pMalc expression plasmid. The fragment of the cDNA corresponding to the intervening segment between these primers was amplified, ethanol precipitated and digested with the appropriate restriction enzyme. The cDNA products were then subjected to PAGE, isolated from the gel and used to ligate appropriately digested pMalc vector. This vector was then used to transform bacteria, and protein production was induced with IPTG. A fusion protein corresponding to the area of TPO encompassed by the primers was then isolated through amylose affinity chromatography. The work was conducted in a manner similar to that described in Specific Example I and Maastricht, J. *et al. J. Clin. Endocrinol. Metab.* 75:121-126 (1992).

Western blots of the 596-611 fusion protein (performed by the protocol set forth above in Specific Example IV), showed that this fusion protein did not contain the epitope, and new primers were used which added four amino acids at either end to produce the region 590-615. Western blots with the resulting fusion protein showed that the AA 590-615 region was recognized by autoantibodies, and could inhibit binding to the whole AA 517-633 fragment. In cross-amplification experiments, it was shown that the 592-615 fragment maintained the epitope whereas the 590-611 fragment did not, thereby documenting that the carboxyl limit of the binding site was located between amino acids 611 and 615. Subsequent amplification showed that if the peptide C-terminus was at an amino acid less than 613, the binding was lost. Together, this localized the epitope binding site between amino acids 592 and 613.

Discussion

The localized binding site between AA 592 and 613 is unique in that it shows strong immunology between the other peroxidase enzymes (meyloperoxidase and lactoperoxidase) with divergent sequences primarily between amino acids 602 and 615. In addition, it is almost entirely homologous between human, porcine and rat TPO with only two non-conserved substitutions within the whole coding region. Thus, it appears that this segment is unique for thyroid peroxidase and does not share sufficient immunology with any of the other peroxidase enzymes to account for cross reactivity. Binding studies with meyloperoxidase and lactoperoxidase also fail to show binding activity in antibodies affinity purified using AA 590-615 peptides. Previously, antibodies against thyroid peroxidase have been shown to cross-react with these two other enzymes, thereby confusing diagnosis. The present invention, however, specifies a region containing an autoantibody epitope that is unique and specific for

thyroid peroxidase. Having now defined this epitope to 22 amino acids, it can be manufactured synthetically and provide specificity in the diagnosis of autoantibodies to thyroid peroxidase and Hashimoto's disease and for use in immunotherapy.

Those skilled in the art can appreciate from the foregoing description that the broad teachings of the present invention can be implemented in a variety of forms. Therefore, while this invention has been described in connection with particular examples thereof, the true scope of the invention should not be so limited since other modifications will become apparent upon a study of the drawings, specification and following claims. All applications and publications cited herein are incorporated by reference. It will be appreciated that the publications cited herein merely reflect the current state of the art and the skills and methodology attendant to those skilled in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Baker, Jr., James R.
Koenig, Ronald J.

(ii) TITLE OF INVENTION: Thyroid Peroxidase Epitopic Regions

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Harness, Dickey & Pierce

(B) STREET: P.O. Box 828

(C) CITY: Bloomfield Hills

(D) STATE: MI

(E) COUNTRY: USA

(F) ZIP: 48303

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lewak, Anna M.

(B) REGISTRATION NUMBER: 33006

(C) REFERENCE/DOCKET NUMBER: 2115-00658PPA

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (313) 641-1600

(B) TELEFAX: (313) 641-0270

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 933 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(D) DEVELOPMENTAL STAGE: Mature

(F) TISSUE TYPE: Thyroid gland (from people with Grave's disease)

(vii) IMMEDIATE SOURCE:

(B) CLONE: phTPO-2.8

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Kimura, S.
Kotani, T.
McBride, O. W.

Umeki, K.
Nakayama, T.
Ohtaki, S.
Hirai, K.

(B) TITLE: Human thyroid peroxidase: Complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 84

(F) PAGES: 5555-5559

(G) DATE: 1987

(K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 3048

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Arg Ala Leu Ala Val Leu Ser Val Thr Leu Val Met Ala Cys Thr
1               5                   10                  15

Glu Ala Phe Phe Pro Phe Ile Ser Arg Gly Lys Glu Leu Leu Trp Gly
            20                  25                  30

Lys Pro Glu Glu Ser Arg Val Ser Ser Val Leu Glu Glu Ser Lys Arg
        35                  40                  45

Leu Val Asp Thr Ala Met Tyr Ala Thr Met Gln Arg Asn Leu Lys Lys
    50                  55                  60

Arg Gly Ile Leu Ser Pro Ala Gln Leu Leu Ser Phe Ser Lys Leu Pro
65                  70                  75                  80

Glu Pro Thr Ser Gly Val Ile Ala Arg Ala Ala Glu Ile Met Glu Thr
                85                  90                  95

Ser Ile Gln Ala Met Lys Arg Lys Val Asn Leu Lys Thr Gln Gln Ser
            100                 105                 110

Gln His Pro Thr Asp Ala Leu Ser Glu Asp Leu Leu Ser Ile Ile Ala
        115                 120                 125

Asn Met Ser Gly Cys Leu Pro Tyr Met Leu Pro Pro Lys Cys Pro Asn
    130                 135                 140

Thr Cys Leu Ala Asn Lys Tyr Arg Pro Ile Thr Gly Ala Cys Asn Asn
145                 150                 155                 160

Arg Asp His Pro Arg Trp Gly Ala Ser Asn Thr Ala Leu Ala Arg Trp
                165                 170                 175
```

```
Leu Pro Pro Val Tyr Glu Asp Gly Phe Ser Gln Pro Arg Gly Trp Asn
            180                 185                 190

Pro Gly Phe Leu Tyr Asn Gly Phe Pro Leu Pro Pro Val Arg Glu Val
        195                 200                 205

Thr Arg His Val Ile Gln Val Ser Asn Glu Val Val Thr Asp Asp Asp
    210                 215                 220

Arg Tyr Ser Asp Leu Leu Met Ala Trp Gly Gln Tyr Ile Asp His Asp
225                 230                 235                 240

Ile Ala Phe Thr Pro Gln Ser Thr Ser Lys Ala Ala Phe Gly Gly Gly
                245                 250                 255

Ala Asp Cys Gln Met Thr Cys Glu Asn Gln Asn Pro Cys Phe Pro Ile
            260                 265                 270

Gln Leu Pro Glu Glu Ala Arg Pro Ala Ala Gly Thr Ala Cys Leu Pro
        275                 280                 285

Phe Tyr Arg Ser Ser Ala Ala Cys Gly Thr Gly Asp Gln Gly Ala Leu
    290                 295                 300

Phe Gly Asn Leu Ser Thr Ala Asn Pro Arg Gln Gln Met Asn Gly Leu
305                 310                 315                 320

Thr Ser Phe Leu Asp Ala Ser Thr Val Tyr Gly Ser Ser Pro Ala Leu
                325                 330                 335

Glu Arg Gln Leu Arg Asn Trp Thr Ser Ala Glu Gly Leu Leu Arg Val
            340                 345                 350

His Ala Arg Leu Arg Asp Ser Gly Arg Ala Tyr Leu Pro Phe Val Pro
        355                 360                 365

Pro Arg Arg Pro Ala Ala Cys Ala Pro Glu Pro Gly Ile Pro Gly Glu
    370                 375                 380

Thr Arg Gly Pro Cys Phe Leu Ala Gly Asp Gly Arg Ala Ser Glu Val
385                 390                 395                 400

Pro Ser Leu Thr Ala Leu His Thr Leu Trp Leu Arg Glu His Asn Arg
                405                 410                 415

Leu Ala Ala Ala Leu Lys Ala Leu Asn Ala His Trp Ser Ala Asp Ala
            420                 425                 430

Val Tyr Gln Glu Ala Arg Lys Val Val Gly Ala Leu His Gln Ile Ile
        435                 440                 445

Thr Leu Arg Asp Tyr Ile Pro Arg Ile Leu Gly Pro Glu Ala Phe Gln
    450                 455                 460
```

```
Gln Tyr Val Gly Pro Tyr Glu Gly Tyr Asp Ser Thr Ala Asn Pro Thr
465                 470                 475                 480

Val Ser Asn Val Phe Ser Thr Ala Ala Phe Arg Phe Gly His Ala Thr
                485                 490                 495

Ile His Pro Leu Val Arg Arg Leu Asp Ala Ser Phe Gln Glu His Pro
            500                 505                 510

Asp Leu Pro Gly Leu Trp Leu His Gln Ala Phe Phe Ser Pro Trp Thr
        515                 520                 525

Leu Leu Arg Gly Gly Gly Leu Asp Pro Leu Ile Arg Gly Leu Leu Ala
    530                 535                 540

Arg Pro Ala Lys Leu Gln Val Gln Asp Gln Leu Met Asn Glu Glu Leu
545                 550                 555                 560

Thr Glu Arg Leu Phe Val Leu Ser Asn Ser Ser Thr Leu Asp Leu Ala
                565                 570                 575

Ser Ile Asn Leu Gln Arg Gly Arg Asp His Gly Leu Pro Gly Tyr Asn
            580                 585                 590

Glu Trp Arg Glu Phe Cys Gly Leu Pro Arg Leu Glu Thr Pro Ala Asp
        595                 600                 605

Leu Ser Thr Ala Ile Ala Ser Arg Ser Val Ala Asp Lys Ile Leu Asp
    610                 615                 620

Leu Tyr Lys His Pro Asp Asn Ile Asp Val Trp Leu Gly Gly Leu Ala
625                 630                 635                 640

Glu Asn Phe Leu Pro Arg Ala Arg Thr Gly Pro Leu Phe Ala Cys Leu
                645                 650                 655

Ile Gly Lys Gln Met Lys Ala Leu Arg Asp Gly Asp Trp Phe Trp Trp
            660                 665                 670

Glu Asn Ser His Val Phe Thr Asp Ala Gln Arg Arg Glu Leu Glu Lys
        675                 680                 685

His Ser Leu Ser Arg Val Ile Cys Asp Asn Thr Gly Leu Thr Arg Val
    690                 695                 700

Pro Met Asp Ala Phe Gln Val Gly Lys Phe Pro Glu Asp Phe Glu Ser
705                 710                 715                 720

Cys Asp Ser Ile Pro Gly Met Asn Leu Glu Ala Trp Arg Glu Thr Phe
                725                 730                 735

Pro Gln Asp Asp Lys Cys Gly Phe Pro Glu Ser Val Glu Asn Gly Asp
            740                 745                 750
```

Phe Val His Cys Glu Glu Ser Gly Arg Arg Val Leu Val Tyr Ser Cys
755 760 765

Arg His Gly Tyr Glu Leu Gln Gly Arg Glu Gln Leu Thr Cys Thr Gln
770 775 780

Glu Gly Trp Asp Phe Gln Pro Pro Leu Cys Lys Asp Val Asn Glu Cys
785 790 795 800

Ala Asp Gly Ala His Pro Pro Cys His Ala Ser Ala Arg Cys Arg Asn
805 810 815

Thr Lys Gly Gly Phe Gln Cys Leu Cys Ala Asp Pro Tyr Glu Leu Gly
820 825 830

Asp Asp Gly Arg Thr Cys Val Asp Ser Gly Arg Leu Pro Arg Ala Thr
835 840 845

Trp Ile Ser Met Ser Leu Ala Ala Leu Leu Ile Gly Gly Phe Ala Gly
850 855 860

Leu Thr Ser Thr Val Ile Cys Arg Trp Thr Arg Thr Gly Thr Lys Ser
865 870 875 880

Thr Leu Pro Ile Ser Glu Thr Gly Gly Gly Thr Pro Glu Leu Arg Cys
885 890 895

Gly Lys His Gln Ala Val Gly Thr Ser Pro Gln Arg Ala Ala Ala Gln
900 905 910

Asp Ser Glu Gln Glu Ser Ala Gly Met Glu Gly Arg Asp Thr His Arg
915 920 925

Leu Pro Arg Ala Leu
930

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3048 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(D) DEVELOPMENTAL STAGE: Mature

(F) TISSUE TYPE: Thyroid gland (from patients with Grave's disease)

(vii) IMMEDIATE SOURCE:

(B) CLONE: phTPO-2.8

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 2pter-q11

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Kimura, S.
Kotani, T.
McBride, O. W.
Umeki, K.
Nakayama, T.
Ohtaki, S.
Hirai, K.

(B) TITLE: Human thyroid peroxidase: Complete cDNA and protein sequence, chromosome mapping, and identification of two alternately spliced mRNAs

(C) JOURNAL: Proc. Natl. Acad. Sci. U.S.A.

(D) VOLUME: 84

(F) PAGES: 5555-5559

(G) DATE: 1987

(K) RELEVANT RESIDUES IN SEQ ID NO:2: FROM 1 TO 3048

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATTTCAGAA GAGTTACAGC CGTGAAAATT ACTCAGCAGT GCAGTTGGCT GAGAAGAGGA 60

AAAAAGGTCA GAATGAGAGC GCTCGCTGTG CTGTCTGTCA CGCTGGTTAT GGCCTGCACA 120

GAAGCCTTCT TCCCCTTCAT CTCGAGAGGG AAAGAACTCC TTTGGGGAAA GCCTGAGGAG
180

TCTCGTGTCT CTAGCGTCTT GGAGGAAAGC AAGCGCCTGG TGGACACCGC CATGTACGCC
240

ACGATGCAGA GAAACCTCAA GAAAAGAGGA ATCCTTTCTC CAGCTCAGCT TCTGTCTTTT
300

TCCAAACTTC CTGAGCCAAC AAGCGGAGTG ATTGCCCGAG CAGCAGAGAT AATGGAAACA
360

TCAATACAAG CGATGAAAAG AAAAGTCAAC CTGAAAACTC AACAATCACA GCATCCAACG
420

GATGCTTTAT CAGAAGATCT GCTGAGCATC ATTGCAAACA TGTCTGGATG TCTCCCTTAC
480

ATGCTGCCCC CAAAATGCCC AAACACTTGC CTGGCGAACA AATACAGGCC CATCACAGGA
540

GCTTGCAACA ACAGAGACCA CCCCAGATGG GGCGCCTCCA ACACGGCCCT GGCACGATGG
600

CTCCCTCCAG TCTATGAGGA CGGCTTCAGT CAGCCCCGAG GCTGGAACCC CGGCTTCTTG
660

TACAACGGGT TCCCACTGCC CCCGGTCCGG GAGGTGACAA GACATGTCAT TCAAGTTTCA
720

AATGAGGTTG TCACAGATGA TGACCGCTAT TCTGACCTCC TGATGGCATG GGGACAATAC
780

ATCGACCACG ACATCGCGTT CACACCACAG AGCACCAGCA AAGCTGCCTT CGGGGGAGGG
840

GCTGACTGCC AGATGACTTG TGAGAACCAA AACCCATGTT TTCCCATACA ACTCCCGGAG
900

GAGGCCCGGC CGGCCGCGGG CACCGCCTGT CTGCCCTTCT ACCGCTCTTC GGCCGCCTGC
960

GGCACCGGGG ACCAAGGCGC GCTCTTTGGG AACCTGTCCA CGGCCAACCC GCGGCAGCAG
1020

ATGAACGGGT TGACCTCGTT CCTGGACGCG TCCACCGTGT ATGGCAGCTC CCCGGCCCTA
1080

GAGAGGCAGC TGCGGAACTG GACCAGTGCC GAAGGGCTGC TCCGCGTCCA CGCGCGCCTC
1140

CGGGACTCCG GCCGCGCCTA CCTGCCCTTC GTGCCGCCAC GGCGGCCTGC GGCCTGTGCG
1200

CCCGAGCCCG GCATCCCCGG AGAGACCCGC GGGCCCTGCT TCCTGGCCGG AGACGGCCGC 1260

GCCAGCGAGG TCCCCTCCCT GACGGCACTG CACACGCTGT GGCTGCGCGA GCACAACCGC 1320

CTGGCCGCGG CGCTCAAGGC CCTCAATGCG CACTGGAGCG CGGACGCCGT GTACCAGGAG 1380

GCGCGCAAGG TCGTGGGCGC TCTGCACCAG ATCATCACCC TGAGGGATTA CATCCCCAGG 1440

ATCCTGGGAC CCGAGGCCTT CCAGCAGTAC GTGGGTCCCT ATGAAGGCTA TGACTCCACC 1500

GCCAACCCCA CTGTGTCCAA CGTGTTCTCC ACAGCCGCCT TCCGCTTCGG CCATGCCACG 1560

ATCCACCCGC TGGTGAGGAG GCTGGACGCC AGCTTCCAGG AGCACCCCGA CCTGCCCGGG 1620

CTGTGGCTGC ACCAGGCTTT CTTCAGCCCA TGGACATTAC TCCGTGGAGG TGGTTTGGAC 1680

CCACTAATAC GAGGCCTTCT TGCAAGACCA GCCAAACTGC AGGTGCAGGA TCAGCTGATG 1740

AACGAGGAGC TGACGGAAAG GCTCTTTGTG CTGTCCAATT CCAGCACCTT GGATCTGGCG 1800

TCCATCAACC TGCAGAGGGG CCGGGACCAC GGGCTGCCAG GTTACAATGA GTGGAGGGAG 1860

TTCTGCGGCC TGCCTCGCCT GGAGACCCCC GCTGACCTGA GCACAGCCAT CGCCAGCAGG 1920

AGCGTGGCCG ACAAGATCCT GGACTTGTAC AAGCATCCTG ACAACATCGA TGTCTGGCTG 1980

GGAGGCTTAG CTGAAAACTT CCTCCCCAGG GCTCGGACAG GGCCCCTGTT TGCCTGTCTC 2040

ATTGGGAAGC AGATGAAGGC TCTGCGGGAT GGTGACTGGT TTTGGTGGGA GAACAGCCAC 2100

GTCTTCACGG ATGCACAGAG GCGTGAGCTG GAGAAGCACT CCCTGTCTCG GGTCATCTGT 2160

GACAACACTG GCCTCACCAG GGTGCCCATG GATGCCTTCC AAGTCGGCAA ATTCCCTGAA 2220

GACTTTGAGT CTTGTGACAG CATCCCTGGC ATGAACCTGG AGGCCTGGAG GGAAACCTTT 2280

CCTCAAGACG ACAAGTGTGG CTTCCCAGAG AGCGTGGAGA ATGGGGACTT TGTGCACTGT
2340

GAGGAGTCTG GGAGGCGCGT GCTGGTGTAT TCCTGCCGGC ACGGGTATGA GCTCCAAGGC
2400

CGGGAGCAGC TCACTTGCAC CCAGGAAGGA TGGGATTTCC AGCCTCCCCT CTGCAAAGAT
2460

GTGAACGAGT GTGCAGACGG TGCCCACCCC CCCTGCCACG CCTCTGCGAG GTGCAGAAAC
2520

ACCAAAGGCG GCTTCCAGTG TCTCTGCGCG GACCCCTACG AGTTAGGAGA CGATGGGAGA
2580

ACCTGCGTAG ACTCCGGGAG GCTCCCTCGG GCGACTTGGA TCTCCATGTC GCTGGCTGCT
2640

CTGCTGATCG GAGGCTTCGC AGGTCTCACC TCGACGGTGA TTTGCAGGTG GACACGCACT
2700

GGCACTAAAT CCACACTGCC CATCTCGGAG ACAGGCGGAG GAACTCCCGA GCTGAGATGC
2760

GGAAAGCACC AGGCCGTAGG GACCTCACCG CAGCGGGCCG CAGCTCAGGA CTCGGAGCAG
2820

GAGAGTGCTG GGATGGAAGG CCGGGATACT CACAGGCTGC CGAGAGCCCT CTGAGGGCAA
2880

AGTGGCAGGA CACTGCAGAA CAGCTTCATG TTCCCAAAAT CACCGTACGA CTCTTTTCCA
2940

AACACAGGCA AATCGGAAAT CAGCAGGACG ACTGTTTTCC CAACACGGGT AAATCTAGTA
3000

CCATGTCGTA GTTACTCTCA GGCATGGATG AATAAATGTT ATAGCTGC
3048

WE CLAIM:

1. A method of screening for the presence of autoantibody to thyroid peroxidase in a sample comprising the steps of:

(a) providing the sample to be screened;

(b) providing a thyroid peroxidase reagent comprising a substantially pure thyroid peroxidase epitopic peptide having an amino acid sequence selected from residue 456 to 933 of the Sequence of SEQ ID NO. 1 or an epitopic fragment thereof;

(c) providing means for detecting binding of the epitopic peptide to autoantibody;

(d) contacting the autoantibody with the thyroid peroxidase reagent under conditions favorable for binding; and

(e) employing means for detecting the binding of the epitopic peptide to the autoantibody to screen for its presence.

2. The method of Claim 1, wherein the sample comprises biological fluid or tissue potentially containing an autoantibody.

3. The method of Claim 1, wherein the sequence of the epitopic fragment consists essentially of from residue 517 to 630.

4. The method of Claim 1, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

5. The method of Claim 1, wherein the sequence of the epitopic fragment consists essentially of from residue 592 to 613.

6. The method of Claim 2, wherein the sample comprises serum.

7. A method of screening for autoimmune thyroiditis in a patient comprising the steps of:

(a) providing a biological tissue or fluid sample potentially containing an autoantibody from the patient;

(b) providing a thyroid peroxidase epitopic reagent comprising a substantially pure thyroid peroxidase epitopic peptide having an amino acid sequence from residue 517 to 630 or residue 633 to 933 of the sequence of SEQ ID NO. 1 or an epitopic fragment thereof;

(c) providing means for detecting binding of autoantibody to the epitopic peptide;

(d) contacting the thyroid peroxidase epitopic reagent and autoantibody under conditions favorable for binding; and

(e) employing means for detecting the binding of the autoantibody to the epitopic peptide to screen for the presence of autoimmune thyroiditis.

8. A method of treating Hashimoto's disease in a patient having a population of unbound autoantibody to a thyroid peroxidase epitope, comprising the steps of:

(a) selecting a substantially pure thyroid peroxidase epitopic peptide having binding equivalence to native thyroid peroxidase epitope;

(b) providing the peptide in a biologically compatible form suitable for administration; and

(c) administering the peptide to the patient in an amount sufficient to competitively bind substantially all the unbound autoantibody.

9. A method of treating Hashimoto's disease in a patient, wherein the patient has a population of thyroid peroxidase epitope and autoantibody thereto, comprising the steps of:

(a) selecting an autoantibody mimic capable of binding thyroid peroxidase native epitope without adversely affecting thyroid peroxidase function;

(b) providing the autoantibody mimic in a biologically compatible form suitable for administration; and

(c) administering the autoantibody mimic to the patient in an amount sufficient to bind substantially all of the thyroid peroxidase epitopes.

10. A method for inducing or stimulating an immune response to thyroid peroxidase in a population of cells comprising the steps of:

(a) providing the population of cells;

(b) providing a thyroid peroxidase reagent comprising a substantially pure thyroid peroxidase epitopic peptide consisting essentially of an amino acid sequence of from residue 465 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof; and

(c) treating the population of cells with the thyroid peroxidase reagent in an amount sufficient and for a time sufficient to induce or stimulate the immune response.

11. The method of Claim 10, wherein the step of providing the population of cells further comprises the step of harvesting the cells from a patient and the method further comprises the step of reintroducing at least a portion of the population after treatment with thyroid peroxidase reagent.

12. A method of stimulating the immune response to thyroid peroxidase in a recipient comprising the steps of:

(a) providing a vaccine comprising an immunostimulatory reagent further comprising a substantially pure thyroid peroxidase epitopic peptide consisting essentially of an amino acid sequence of from residue 456 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof, in a biologically compatible form suitable for administration; and

(b) administering the vaccine in an amount sufficient to stimulate the response.

13. The method of Claim 12, wherein the sequence of the epitopic fragment consists essentially of from residue 517 to 630.

14. The method of Claim 12, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

15. The method of Claim 12, wherein the sequence of the epitopic fragment consists essentially of from residue 592 to 613.

16. The method of Claim 12, wherein the vaccine further comprises an adjuvant.

17. A vaccine for inducing an immune response to thyroid peroxidase, the vaccine comprising an immunostimulatory reagent further comprising a substantially pure thyroid peroxidase epitopic peptide consisting essentially of a sequence of amino acids of from residue 456 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof.

18. The vaccine of Claim 17, wherein the peptide is obtained from a source selected from the group consisting essentially of recombinant protein, synthetic peptides and native thyroid peroxidase.

19. The vaccine of Claim 17, wherein the sequence of the epitopic fragment consists essentially of from residue 517 to 630.

20. The vaccine of Claim 17, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

21. The vaccine of Claim 17, wherein the sequence of the epitopic fragment consists essentaily of from residue 592 to 613.

22. The vaccine of Claim 17, wherein the vaccine further comprises an adjuvant.

23. The vaccine of Claim 17, wherein the vaccine is for Hashimoto's disease.

24. The vaccine of Claim 17, wherein the vaccine is for thyroid cancer.

25. A substantially pure thyroid peroxidase epitopic peptide consisting essentially of an amino acid sequence of from residue 456 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof.

26. The sequence of Claim 25, wherein the sequence of the epitopic fragment consists essentially of from residue 517 to 630.

27. The sequence of Claim 25, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

28. The sequence of Claim 25, wherein the sequence of the epitopic fragment consists essentially of from residue 592 to 613.

29. The sequence of Claim 25, wherein the peptide is obtained from a source selected from the group consisting essentially of recombinant protein, synthetic peptides and native thyroid peroxidase.

30. A nucleic acid consisting essentially of a DNA or RNA sequence coding for the amino acid sequence or epitopic fragment thereof of Claim 25.

31. A nucleic acid consisting essentially of a DNA or RNA sequence coding for the amino acid sequence or epitopic fragment thereof of Claim 26.

32. A nucleic acid consisting essentially of a DNA or RNA sequence coding for the amino acid sequence or epitopic fragment thereof of Claim 27.

33. A nucleic acid consisting essentially of a DNA or RNA sequence coding for the amino acid sequence or epitopic fragment thereof of Claim 28.

34. A nucleic acid having a sequence complementary to the sequence of Claim 29.

35. A nucleic acid having a sequence complementary to the sequence of Claim 31.

36. A nucleic acid having a sequence complementary to the sequence of Claim 32.

37. A nucleic acid having a sequence complementary to the sequence of Claim 33.

38. A kit for assaying for autoimmune thyroiditis comprising:

(a) a substantially pure thyroid peroxidase epitopic peptide which specifically binds to thyroid peroxidase autoantibody, the peptide having a sequence consisting essentially of an amino acid sequence of from residue 456 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof;

(b) reagent means for detecting the binding of the autoantibody to the epitope; and

(c) the epitopic peptide and reagent means each being present in amounts effective to perform the immunoassay.

39. The kit of Claim 38, wherein the sequence of the epitopic fragment consists essentially of from residue 517 to 630.

40. The kit of Claim 38, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

41. The kit of Claim 38, wherein the sequence of the epitopic fragment consists essentially of from residue 592 to 613.

42. A method of screening for the presence of Hashimoto's disease in a sample comprising the steps of:

(a) providing the sample to be screened;

(b) providing a thyroid peroxidase reagent comprising a substantially pure thyroid peroxidase epitopic peptide having an amino acid sequence consisting essentially of from residue 456 to 933 of SEQ ID NO. 1 or an epitopic fragment thereof;

(c) providing means for detecting binding of the epitopic peptide to autoantibody;

(d) contacting the autoantibody with the thyroid peroxidase reagent under conditions favorable for binding; and

(e) employing means for detecting the binding of the eptitopic peptide to the autoantibody.

43. The method of Claim 42, wherein the sample comprises biological fluid or tissue potentially containing an autoantibody.

44. The method of Claim 42, wherein the sequence of the epitopic fragment consists essentially of from residue 592 to 613.

45. The method of Claim 42, wherein the sequence of the epitopic fragment consists essentially of from residue 633 to 933.

46. A method for treating autoimmune thyroidites in a patient having blood comprising serum having autoantibody specific for thyroid peroxidase, comprising the steps of:

a) removing a portion of blood comprising serum having autoantibody from the patient;
b) providing a thyroid peroxidase epitopic reagent comprising a substantially pure thyroid peroxidase epitopic peptide having an amino acid sequence selected from residue 456 to 933 of the Sequence of SEQ ID NO. 1 or an epitopic fragment thereof;
c) contacting the thyroid peroxidase epitopic reagent with the portion of serum having autoantibody under conditions favorable for binding; and
d) reintroducing the portion of serum to the patient.

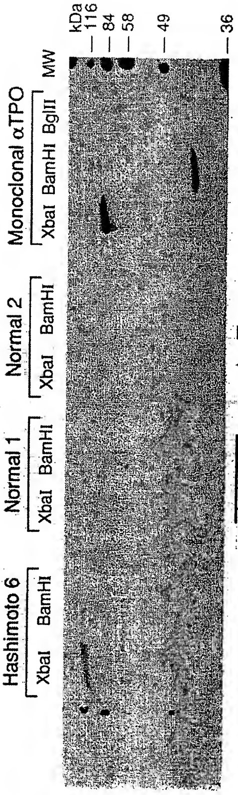

Hashimoto 6
XbaI BamHI
Normal 1
XbaI BamHI
Normal 2
XbaI BamHI
Monoclonal αTPO
XbaI BamHI BglII
MW
kDa
116
84
58
49
36

FIG. 1A.

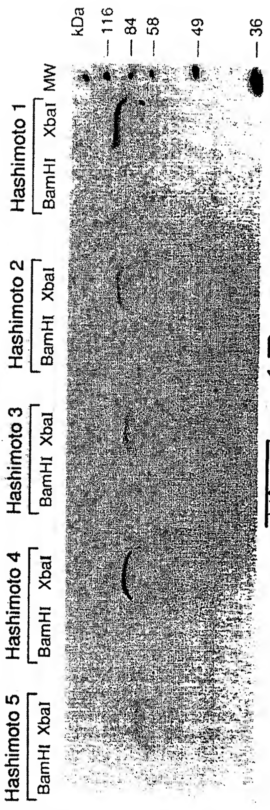
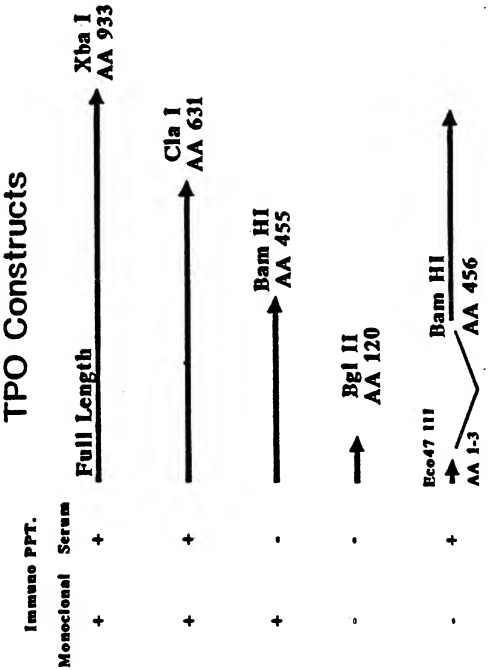

Hashimoto 5
BamHI XbaI
Hashimoto 4
BamHI XbaI
Hashimoto 3
BamHI XbaI
Hashimoto 2
BamHI XbaI
Hashimoto 1
BamHI XbaI
MW
kDa
116
84
58
49
36

FIG. 1B.

Figure 2


TPO Constructs
Immuno PPT.
Monoclonal
Serum
Full Length
Xba I
AA 933
Cla I
AA 631
Bam HI
AA 455
Bgl II
AA 120
Eco47 III
AA 1-3
Bam HI
AA 456

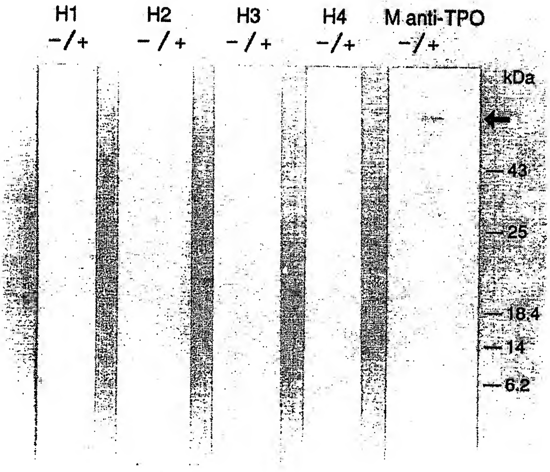


Fig. 3.

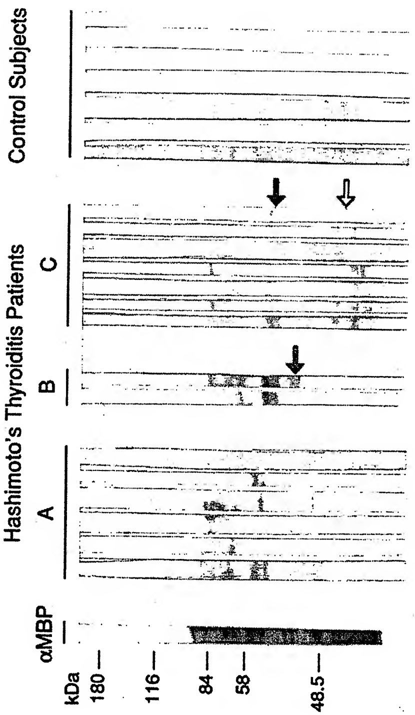
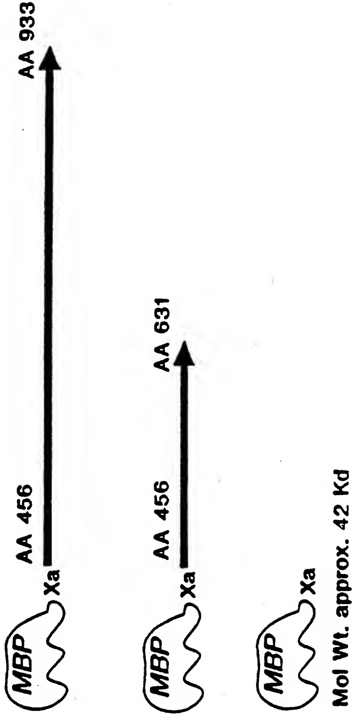

Control Subjects
Hashimoto's Thyroiditis Patients
A
B
C
αMBP
kDa
180
116
84
58
48.5

FIG. 4

Figure 5

TPO-Maltose Binding Protein Fusion Products



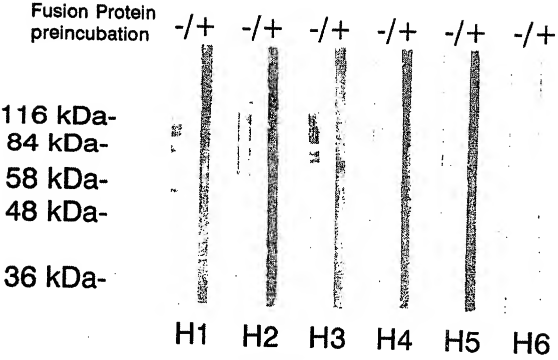

Fusion Protein
preincubation
-/+ -/+ -/+ -/+ -/+ -/+
116 kDa-
84 kDa-
58 kDa-
48 kDa-
36 kDa-
H1 H2 H3 H4 H5 H6

FIG. 8.

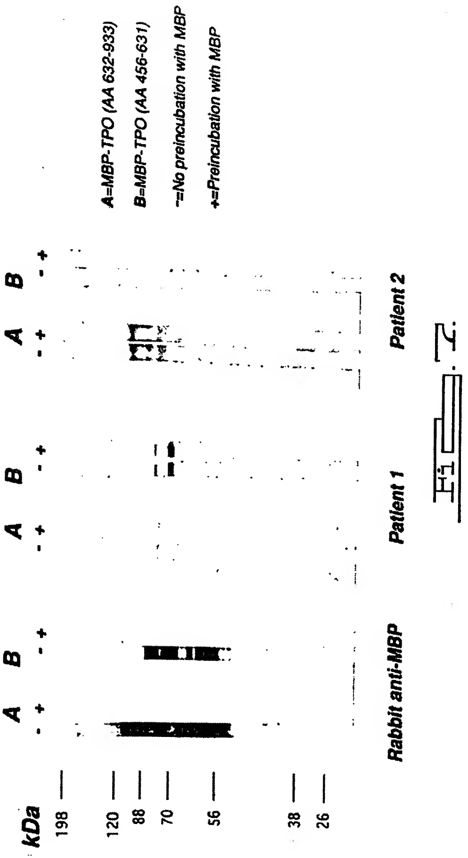
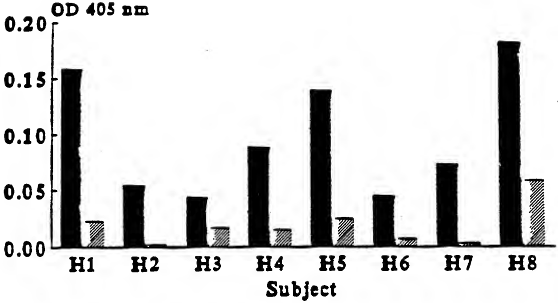

A=MBP-TPO (AA 632-933)
B=MBP-TPO (AA 456-631)
-=No preincubation with MBP
+=Preincubation with MBP
kDa
198
120
88
70
56
38
26
A B A B A B
- + - + - + - + - + - +
Rabbit anti-MBP
Patient 1
Patient 2
FIG. 7

Figure 8



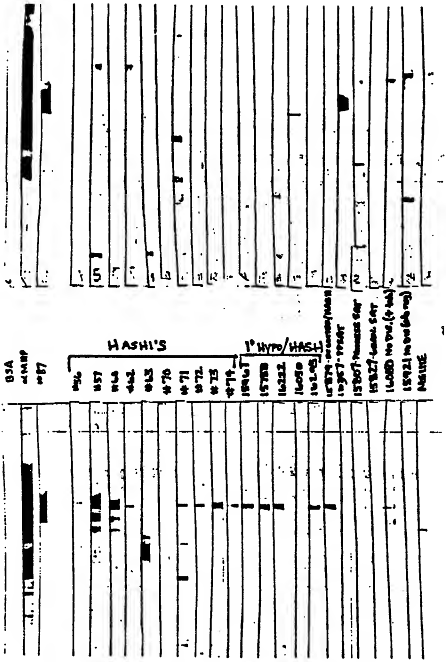


Figure 9A

←AA560-630

Figure 9B

←AA 631-933

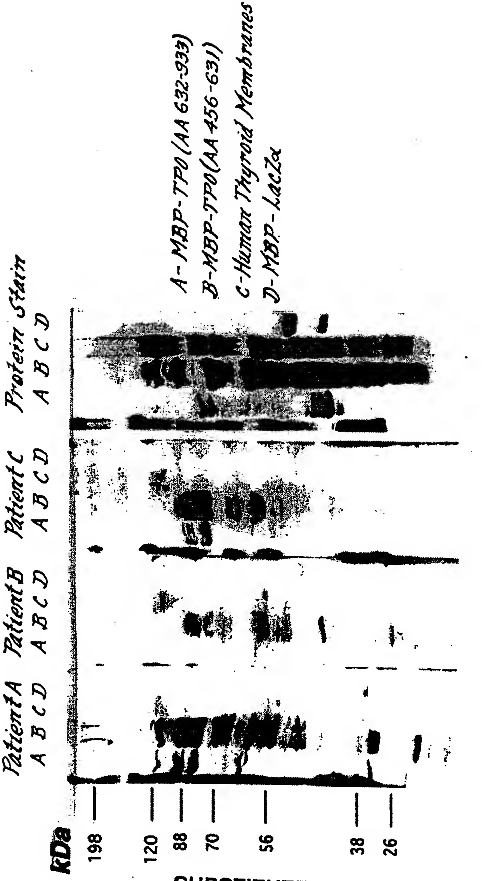

A-MBP-TPO (AA 632-933)
B-MBP-TPO (AA 456-631)
C-Human Thyroid Membranes
D-MBP-LacZα
Protein Stain
A B C D
Patient C
A B C D
Patient B
A B C D
Patient A
A B C D
kDa
198
120
88
70
56
38
26

Fig. 10

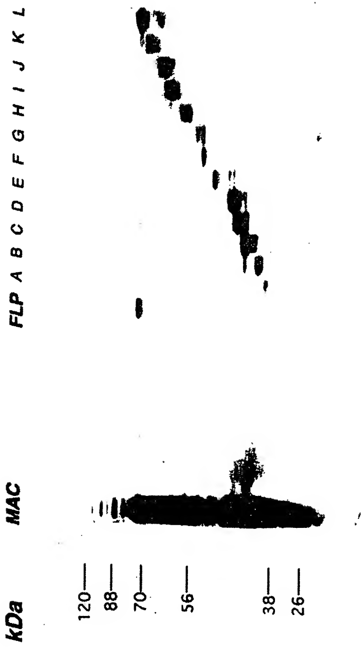


FIG. 11.

Figure 12

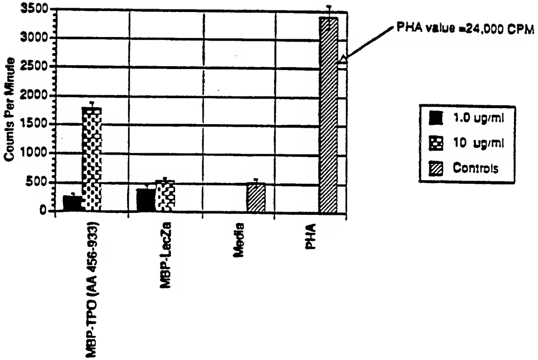

PHA value =24,000 CPM
3500
3000
2500
2000
1500
1000
500
0
Counts Per Minute
1.0 ug/ml
10 ug/ml
Controls
MBP-TPO (AA 456-933)
MBP-LacZa
Media
PHA

Figure 13A

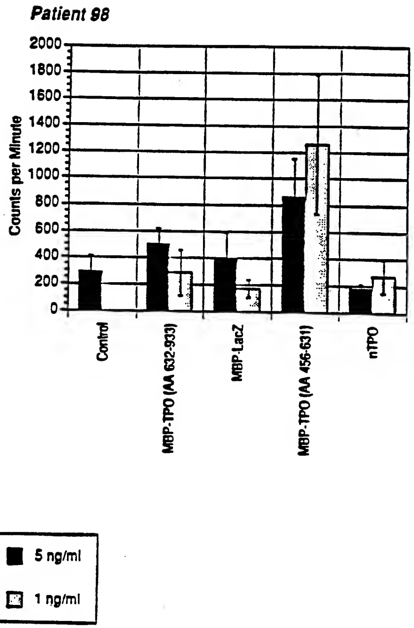

Patient 98
Counts per Minute
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Control
MBP-TPO (AA 632-933)
MBP-LacZ
MBP-TPO (AA 456-631)
nTPO
5 ng/ml
1 ng/ml

Figure 13B

Patient 97

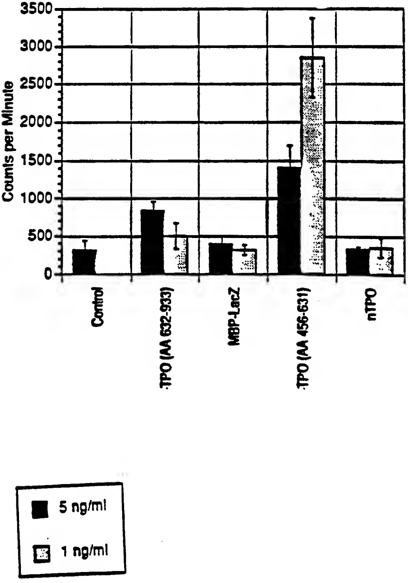
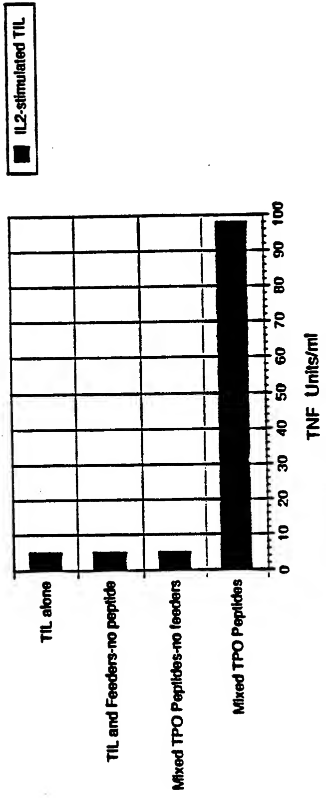


Figure 14


IL2-stimulated TIL
TIL alone
TIL and Feeders-no peptide
Mixed TPO Peptides-no feeders
Mixed TPO Peptides
0
10
20
30
40
50
60
70
80
90
100
TNF Units/ml

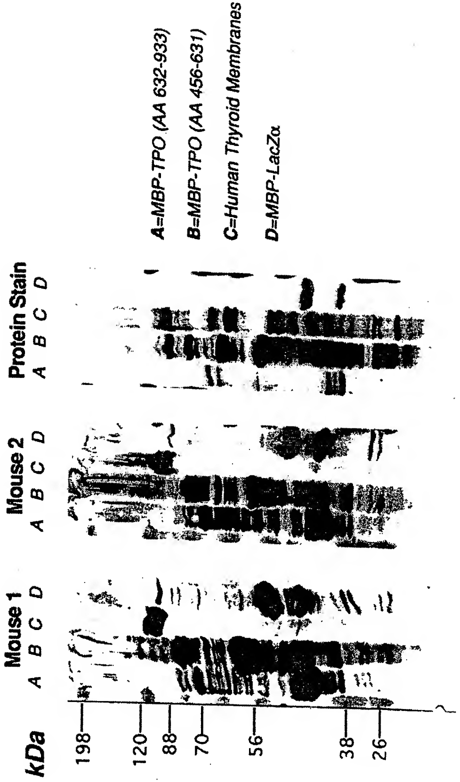
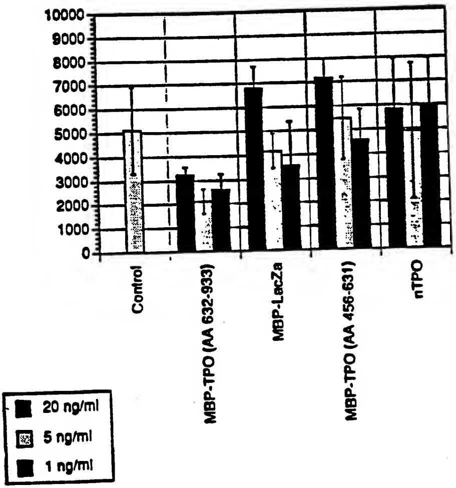

A=MBP-TPO (AA 632-933)
B=MBP-TPO (AA 456-631)
C=Human Thyroid Membranes
D=MBP-LacZα
Protein Stain
A B C D
Mouse 2
A B C D
Mouse 1
A B C D
kDa
198
120
88
70
56
38
26

FIG. 15.

Figure 16


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
MBP-TPO (AA 632-933)
MBP-LacZa
MBP-TPO (AA 456-631)
nTPO
20 ng/ml
5 ng/ml
1 ng/ml

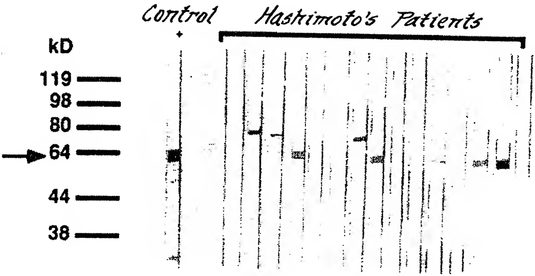


FIG. 17A.

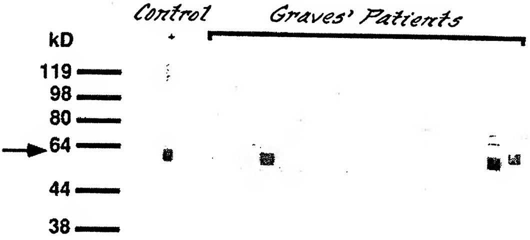
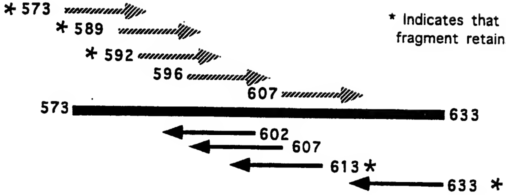


FIG. 17B.

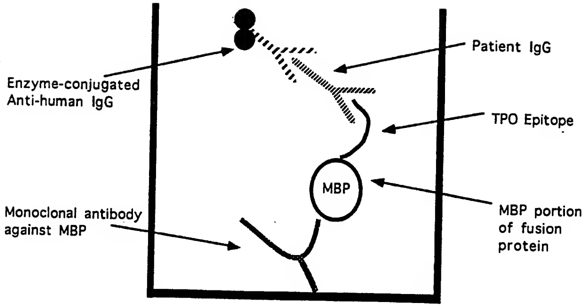
Figure 18



592	N E W R E F C G L P R L E T P A D L S T A I	613	TPO
684	N A W R R F C G L P Q P E T V G Q L Q T V L	707	MPO
564	N S W R G F C G L S Q P K T L K G L Q T V L	589	LPO

592	N E W R E F C G L P R L E T P A D L S T A I	human TPO
590	N E W R E F C G L S R L E T W A D L S A A T	porcine TPO
580	N E W R E F C G L S R L D T G A E L N K A T	rat TPO
580	N E W R E F C G L S R L E T P A E L N K A I	mouse TPO

Figure 19


Enzyme-conjugated
Anti-human IgG
Patient IgG
TPO Epitope
MBP
MBP portion
of fusion
protein
Monoclonal antibody
against MBP

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03837

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :530/326, 395, 854; 435/7.4, 7.92, 975; 436/811
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/326, 395, 854; 435/7.4, 7.92, 975; 436/811

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS, DERWENT WORLD PATENT INDEX, PIR 33, SWISS-PROT 23, A-GENESEQ 8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM, Volume 68, No. 6, issued 1989, Ludgate et al, "Antibodies to Human Thyroid Peroxidase in Autoimmune Thyroid Disease: Studies with a Cloned Recombinant Complementary Deoxyribonucleic Acid Epitope", pages 1091-1096, see entire document.	1,2,25,29, 41,42 3-7, 26-28, 43-44
X Y	WO, A, 91/02061 (Rapaport) 21 February 1991, see entire document, particularly Claims 9-10 and 21-22, and pages 8, 32-35, and 57-63.	1-2,37"B", 41-42 3-7, 38-40, 43-44

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 JULY 1993	14 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JAMES L. GRUN, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03837

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KLEIN, "NATURAL HISTORY OF THE MAJOR HISTOCOMPATIBILITY COMPLEX" published 1986 by John Wiley and Sons (N.Y.), see page 235.	6
X Y	JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM, Volume 73, No. 4, issued October 1991, Libert et al, "Thyroperoxidase, but not the Thyrotropin Receptor, Contains Sequential Epitopes Recognized by Autoantibodies in Recombinant Peptides Expressed in the pUEX Vector", pages 857-860, see entire document, especially page 859, Figure 1.	25, 29 3-5, 7, 26-28, 38-40, 43-44
X Y	CLIN. EXP. IMMUNOL., Volume 87, issued January 1992, Zanelli et al, "Use of Recombinant Epitopes to Study the Heterogeneous Nature of the Autoantibodies Against Thyroid Peroxidase in Autoimmune Thyroid Disease", pages 80-86, see entire document, especially page 84, Col 2.	25, 29 3-5, 7, 26-28, 38-40, 43-44
Y	PROC. NATL. ACAD. SCI. USA, Volume 84, issued August 1987, Kimura et al, "Human Thyroid Peroxidase: Complete cDNA and Protein Sequence, Chromosome Mapping, and Identification ot Two Alternately Spliced mRNAs", pages 5555-5559, see page 5557, Figure 2.	3-5, 7, 26-28, 38-40, 43-44
Y	JOURNAL OF IMMUNOLOGICAL METHODS, Volume 102, issued 1987, Geysen et al, "Strategies for Epitope Analysis Using Peptide Synthesis", pages 259-274, see entire document.	5, 28, 40, 43
Y	PHARMACIA P-L BIOCHEMICALS, "MOLECULAR AND CELL BIOLOGY CATALOGUE", published 1992 by Pharmacia P-L Biochemicals (Milwaukee), see pages 144-160.	3-4, 7, 26-27, 38-39, 44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03837

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 37/50; C07K 3/04, 3/02, 7/10, 13/00; C12Q 1/28; G01N 33/564, 33/573

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-7, 25-29, and 37"B"-44, drawn to antigenic peptides of thyroid peroxidase and their use in a method and kit for the detection of anti-thyroid peroxidase autoantibodies classified in Class C 07 K, Subclass 13/00, Class C 12 Q, subclass 1/28, and Class G 01 N, Subclasses 33/564 and 33/573.

II. Claims 8, 10-24, and 45, drawn to therapeutic uses of thyroid peroxidase peptides classified in Class A 61 K, Subclass 37/50.

III. Claim 9 drawn to the therapeutic use of an antibody classified in Class A 61 K, Subclass 39/395.

IV. Claims 30-37"A" drawn to nucleic acids encoding thyroid peroxidase amino acid sequences classified in Class C 07 H, Subclass 15/12 or 17/00.

The inventions of Groups II-IV are not so linked as to form a single general inventive concept with each other or with the Main Invention of Group I. Groups II-IV share no special technical feature with each other or with the Main Invention.

Group II is drawn to the in vivo therapeutic uses of thyroid peroxidase peptides which are unrelated in design or performance to the in vitro assay for the detection of anti-thyroid peroxidase antibodies of the Main Invention. Thus the inventions are alternative methods, i.e. therapy or a diagnostic assay, using thyroid peroxidase peptides and share no special technical feature.

Group III is drawn to the in vivo therapeutic use of anti-thyroid peroxidase antibodies and is unrelated in structure, design, and performance to either the Main Invention or the method of Group II, thus, sharing no special technical feature.

Group IV is drawn to compositions which are structurally unrelated to the peptides or antibodies of the Main Invention and Groups II-III, thus sharing no special technical feature in common with the other inventions.